DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop AF
Commissioner of Patents
P.O. 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Guenther Eckhard declare and state that:

1. I am an inventor listed on U.S. application Serial No. 10/632,187. I am familiar with the prosecution history of this application, particularly the Office Action mailed on April 10, 2009, and have read and understand all the patent references cited by the examiner and applied to reject the claims.

2. I am a knowledgeable expert in the field of pharmaceutical arts, as evidenced by my resume, which is attached as Exhibit A. I hold a doctorate degree in chemistry from the University of Halle-Wittenberg, where I performed a doctoral thesis in Synthetic Organic Chemistry. I also have over 25 years experience in medicinal chemistry, specializing in antitumor drug development, and I am an inventor on over thirty (30) patents in medicinal chemistry and antitumor drugs. I am currently an employee of *A*Eterna Zentaris and hold the title of Vice President, Alliance Management & IP. I have held this title since 2008. Between 2002 and 2008, I was Vice President of Drug Discovery and Preclinical Development. Prior to assuming the role of Vice President, I worked for seventeen years for ASTA Medica AG, the parent company of *A*Eterna Zentaris, in various capacities in the research and development, drug discovery, and preclinical development of antitumor drugs. In view of my education, training and experience, I consider myself qualified to express the opinions stated herein.

3. The present invention claims a method of treating mammary carcinoma comprising administering a combination of an alkylphosphocholine and another antitumor agent.

4. We have surprisingly discovered that the combination of alkylphosphocholines, especially perifosine, with other antitumor agents results in a synergistic therapeutic effect that is useful for treating cancer.

5. In particular, we have discovered that the combination of perifosine with particular antimetabolite antitumor agents results in a synergistic therapeutic effect that is useful for treating mammary carcinoma.

6. The experimental data shown below were generated at my direction and under my supervision in the pharmacology laboratories of AEZS at its Frankfurt site. For the detection of the cytotoxic effect, we used the AlamarBlue assay with 48 hours compound incubation. The experiments were conducted on a Biomek 2000 pipetting station, using constant ratio of two drug combinations, with 1:1.25 dilution steps, and evaluating nine concentrations, with the fifth concentration at about EC40. The data was evaluated using the CalcuSyn Dose-Effect Analysis software. Experimental data within the linear efficacy range was analysed via median effect plot, CI and isobologram analysis.

Experimental Details:

Measurement of the cellular cytotoxic/antiproliferative activity of Perifosine alone and in combination with different antimetabolites is based on the dye Resazurin (Sigma, cat. no. R7017), which exhibits fluorescence change in the appropriate oxidation-reduction range relating to cellular metabolic reduction, (Brigitte Page, *Int. J. Oncology* 3: 473-476 (1993), *see* Exhibit B) yielding a fluorescence signal at 590 nm. The increase of fluorescence at 590 nM is an indicator of cellular viability/cell number.

The cells were seeded in the respective growth medium (media and reagents purchased from Gibco-BRL) in 125 µl per 96 well and were grown for 24h at 37°C/5%CO_2. The cell number shown is adapted for the cell line HCC1806 to generate signals in the linear detection range under the experimental conditions applied.

Cell Line	Source	Cell Number / Well	Volume / Well	Medium
HCC1806 (Breast Cnacer)	ATCC CRL-2235	4,500	125	RPMI 1640 + 10% FCS + 1% PS + 1% HEPES + 1% sodium pyruvate + 2,5% 1M glucose

Dose response analysis of single agents typically started with a concentration of 100 µM based on 10 mM compound stocks in 100% DMSO or water for the antimetabolites and Perifosine, respectively, and was performed in serial dilution steps of 1:2. The compounds were prediluted in cell medium and the final DMSO concentration in compound and control wells was adjusted to 1%. 25 µl of the respective compound dilutions were added per 96 well.

For the analysis of drug combinations, perifosine and the respective antimetabolite were combined in a fixed ratio based on the specific EC_{50} of both compounds in the respective cell line. For a nine point dose response curve serial dilution in 1:1.25 steps was performed, with the fifth data point yielding efficacies around EC_{30}-EC_{40} for both compounds.

After 45h of compound incubation at 37°C/5%CO_2, 15 µl of the Resazurin detection reagent (0.2 mg/mL in DPBS (Gibco, 14190), steril filtered) was added for an additional 3 hours and after a total of 48 hours of compound incubation the cellular metabolic activity was quantified by measurement of fluorescence at 590 nm. Non-treated cells and blank controls w/o cells were set as reference values.

MS EXCEL was used for formating and analysis of data. All data were calculated as % efficacy compared to the mean of the respective negative (non-treated cells) and positive control wells (blank) on each assay plate. For dose response analysis of single agents EC50 values were calculated by using non-linear regression software GraphPad Prism.

The analysis of drug-drug combinations was conducted by using the software CalcuSyn (Biosoft, Cambridge, UK), which yields as classification for the type of combination the combination index (CI) and dose reduction index (DRI) as introduced by Chou et al.

7. Table 1 below shows the concentration of perifosine that exhibits 50% of the maximum drug effect (i.e., EC_{50}). EC_{50} values are used to report antiproliferative data because in most of the cases the effect is not based on a single molecular target, like an enzyme (and accordingly IC_{50} is not used). The EC_{50} value is the molar concentration of a given compound which produces 50% of the maximum possible response (effect) for that compound. For example, Table 1 shows that a 3.753 μM concentration of perifosine produces 50% of the maximum inhibitory effect observed for perifosine in this breast cancer cell line. There is no difference between IC50 and EC50 in terms of value.

Table 1.
Activity of Perifosine in Breast Cancer Cell Line (HCC1806)

Alkylphosphocholine	**EC_{50} [μM]**
Perifosine	3.753

8. Table 2 below shows the concentration of each of the listed individual antimetabolites that exhibits 50% of the maximum drug effect (*i.e.*, EC_{50}). For example, a 0.40 μM concentration of cytarabine produces 50% of the maximum inhibitory effect observed for cytarabine in this breast cancer cell line.

Table 2.
Activity of Individual Antimetabolites in Breast Cancer Cell Line (HCC1806)

Antimetabolite	EC_{50} [μM]
Gemcitabine	~ 0.003-0.005
Cytarabine	0.40
Fludarabine	> Cmax
5-Fluorouracil	20.63

9. The properties of the combinations of alkylphosphocholines with antimetabolites were demonstrated using the combination index method. (*See* Chou, Ting-Chao and Paul Talalay, *Quantitative Analysis Of Dose-Effect Relationships: The Combined Effects Of Multiple Drugs Or Enzyme Inhibitors*, Advances in Enzyme Regulation (1984), 22, 27-55; *See also* Chou, Ting-Chao, *Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method*, Cancer Research (2010), 70(2), 440-446). These references are attached as Exhibits C and D respectively.

The combination index (CI) is one of the most popular methods to evaluate drug-drug interactions, developed by Chou and Talaly in 1984. The method is based on the median-effect equation, derived from the mass-action law principle, and provides algorithms for computer simulation of synergistic or antagonistic effects of drug-drug combinations. The CI is an index to quantitatively depict synergism.

The following table gives the criteria for classification of a particular combination:

- Classification based on Combination Index (CI)
  - CI<0.30: strong synergism
  - CI=0.30-0.70: synergism
  - CI=0.70-0.85: moderate synergism
  - CI=0.85-0.90: slight synergism
  - CI=0.90-1.10: nearly additive
  - CI=1.10-1.20: slight antagonism
  - CI=1.20-1.45: moderate antagonism
  - CI=1.45-3.30: antagonism
  - CI>3.30: strong antagonism

The data in Tables 3 clearly demonstrate that perifosine in combination with the listed antimetabolite antitumor agents produce a useful synergistic effect against breast cancer. In these results, a given EC value corresponds to a distinct mixture of compound A and compound B (extrapolated value from the isobologram analysis). The CI index has the shown calculated value at that particular EC value.

The combinations are synergistic at some concentrations but antagonistic at other concentrations because at high concentrations there can be 100% inhibition of cell proliferation by one of the compounds. Accordingly, the combination cannot demonstrate the benefit of the additional drug. Moreover, the additive effect of two drugs is not simply the sum of the EC values. There is no link between the CI and mode-of-action explanations or predictions.

These combinations are useful despite the fact that the combinations are synergistic at some concentrations but antagonistic at other concentrations because a useful combination demonstrates synergistic effects at a broader range of concentrations, and especially at lower concentrations, which are more relevant in the treatment of humans.

These results are unexpected because one can not foresee which combination of perifosine is useful (synergistic) and which combination is not useful (antagonistic) in a

specific cancer entity based on the activity of individual antimetabolites shown in Table 2 or on the teachings of the prior art.

Table 3.
Combination Index of Perifosine in Breast Cancer Cell Line (HCC1806) with Particular Antimetabolites

Perifosine	Combination Index (CI) at **EC30**	Combination Index (CI) at **EC50**	Combination Index (CI) at **EC75**	Combination Index (CI) at **EC90**	**Classification**
Cytarabine	0.280	0.495	1.143	2.671	Synergism – Slight Antagonsim (<EC90)
Gemcitabine	0.475	0.615	0.874	1.262	Synergism - Moderate Synergism (<EC90)

10. All statements made herein are of my own knowledge and true and all statements on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 05/07, 2010

Dr. Eckhard Guenther

EXHIBIT A

Curriculum Vitae
of
Dr. Eckhard G. Guenther

Personal Details

Dr. Eckhard Gottfried Guenther
born on 05.03.1956 in Greifswald

Education

1962 - 1970	Primary School
1970 - 1974	Secondary School: Gymnasium in Greifswald
1974 - 1979	Study of Chemistry at the Martin-Luther-University of Halle-Wittenberg
February 1979	Diploma examination
1979 - 1980	Military service
1980 - 1984	Doctoral thesis in Synthetic Organic Chemistry at the University of Halle-Wittenberg under the supervision of Prof. M. Augustin
December 1985	Graduation (Dr. rer. nat.), summa cum laude

Professional experiences

1985 - 1986	Scientific coworker, R&D department Medicinal Chemistry, Fahlberg-List, Magdeburg
1986 - 1989	Scientific assistant at the University of Halle-Wittenberg, Institute of Organic Chemistry
1990 - 1992	Head of chem. laboratory, R&D department Medicinal Chemistry, ASTA Medica AG, Frankfurt
1992 - 1993	Group leader planing & controlling, research coordination, R&D, ASTA Medica AG, Frankfurt
1994 - 1997	Head of research coordination, R&D, ASTA Medica AG, Frankfurt
1997 - 2000	Head of Medicinal Chemistry Oncology, and Project Manager New Antitumor Drugs, ASTA Medica AG, Frankfurt

2001 - 2002	Head of Drug Discovery , Zentaris AG, Frankfurt
2002 - 2006	Vice President Drug Discovery, Aeterna Inc., Quebec and Head of Drug Discovery Zentaris GmbH, Frankfurt
2006 - 2008	Head of Drug Discovery and Preclinical Development, AeternaZentaris Inc., Quebec and Zentaris GmbH Frankfurt
since December 2008	Vice President Alliance Management and Intellectual Property, Aeterna Inc., Quebec and Zentaris GmbH Frankfurt

Responsibility:

- Project management and coordination
- Controlling budget and capacity
- Alliance management
- Out-licensing activities
- Project exploitation
- Patent department
- International patent filing and maintenance
- International trademarks
- Research collaborations
- Project documentation

Track record

- Cetrorelix, supportive work, process development, mode-of-action, BPH, phase III
- Impavido, process development, pharmacology, anti-leishmania drug, on the market
- Lobaplatin, co-inventor, process development, pharmacology, cancer, in China on the market
- Perifosine, medicinal chemistry, pharmacology, process development, cancer, phase II
- Ozarelix, co-inventor, endocrinology, phase II
- AEZS-108, process development, mode-of-action, cancer, phase II
- AEZS-112, co-inventor, preclinical development, cancer, phase I
- AEZS-115, co-inventor, endocrinology, preclinical development
- AEZS-126, co-inventor, cancer, preclinical development

Scientific experiences

- Modern methods in drug discovery & development
- Oncology, Endocrinology, Antiinfectiva
- Signaltransduction, GPCRs
- Medicinal Chemistry
- Natural products, nucleic acid & peptide chemistry
- Chemistry of heterocyclic compounds

- Discovery & development of NCE's in oncology and endocrinology
- Hit-to-Lead process and lead optimization
- In-vitro & in-vivo Pharmacology
- Compound library & handling
- Computational chemistry
- ADME and pharmacokinetic
- Tox & safety pharmacology
- API supply (GMP, non-GMP)
- Preparation and approval of regulatory documents

Business experiences

- Organization and management of large scientific departments
- Project management and R&D controlling
- Membership of several project teams and steering committees
- Organization of scientific cooperations with university groups
- Project leadership
- Management of alliances with international pharmaceutical companies
- Hands-on experience within an US biotech company

Other experiences

- Membership in an accreditation agency (ASIIN) for the reorganization of the university education in Germany (FA 10, life sciences)
- Organization and controlling of projects with funding from the German government and EU organizations

Membership

- American Chemical Society (ACS)
- American Association for Cancer Research (AACR)
- Gesellschaft Deutscher Chemiker (GDCh)

Publications

- papers in organic chemistry, medicinal chemistry, drug discovery
- poster and oral presentations
- 30 patents in medicinal chemistry, antitumor drugs etc.

Dr. Eckhard Guenther Frankfurt, June 15th, 2009

EXHIBIT B

International Journal of Oncology

ISSN 1019-6439

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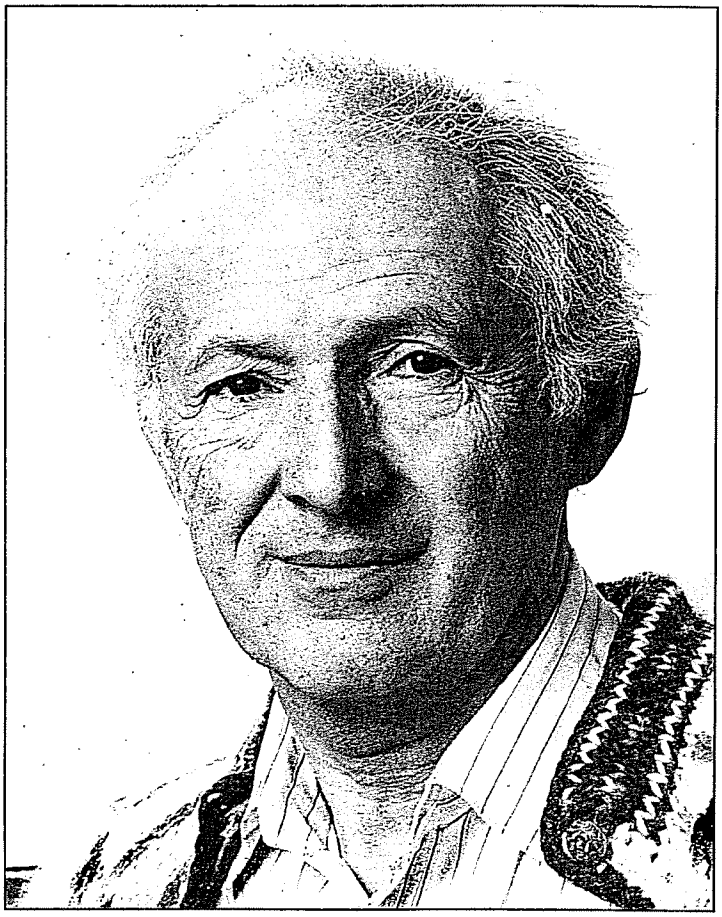
VOLUME 3, NUMBER 3, SEPTEMBER 1993



INTERNATIONAL JOURNAL OF ONCOLOGY 3: 473-476, 1993

A new fluorometric assay for cytotoxicity measurements *in vitro*

BRIGITTE PAGÉ, MICHEL PAGÉ and CHRISTIANE NOËL

Department of Biochemistry, Faculty of Medicine, Université Laval, Québec G1K 7P4, Canada

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Abstract. Cytotoxicity testing of anticancer drugs requires techniques which are sensitive, reproducible and applicable to large scale testing using automated instruments. These assays are presently performed with end point staining of cell proteins with dyes, viability stains or energy dependent of substrates such as MTT or XTT. Although reliable, these assays are not sensitive enough, too expensive for large scale screening or they use reagents that may be harmful for personnel or equipment. We describe, the use of Alamar Blue, a new non fluorescent substrate, which after reduction in living cells, yields a very strong fluorescent product. Using the automated fluorescence plate reader Cytofluor, we have evaluated the various parameters such as substrate concentration, time and volume of incubation with respect to linearity and lower limit of detection. We found that for a two hour assay, this new non toxic substrate could detect as low as 200 cells per well with a useful measurement range up to 20,000 cells per well. The fluorescent assay is more than ten times as sensitive as the colorimetric assay. When the cytotoxicity of daunorubicin was measured with this assay and compared to the XTT formazan assay we found comparable IC_{50} values but this new assay was more economical and results are obtained in two hours as compared to four hours for the formazan assay. This new economical and versatile assay could be used with advantage for large scale *in vitro* screening of anticancer drugs and other cytotoxic agents.

Introduction

In 1955, Puck *et al* reported a method for determining chemosensivity of human tumors *in vitro* (1). Colony counting, however does not always reflect cell growth because of the large variations in colony diameter. To circumvent this variable a semiautomated method in which the surface occupied by the colonies was integrated has already been reported (2). Dye exclusion techniques (3) are widely used for cells growing in suspension, but for low cell numbers the method is not applicable. Assays utilising uptake of radioactive precursors are not always practical and potentially hazardous for large scale routine cytotoxicity measurements. Mosmann has already reported a colorimetric assay based on the reduction of tetrazolium derivative by various dehydrogenases (3,4). In this assay a colored insoluble tetrazolium salt is produced which could be solubilized by alcohol or dimethyl sulfoxide. A series of new tetrazolium salts have been developed which, upon metabolic reduction by viable cells, yield aqueous-soluble formazans (5,6). We have previously reported the optimization of the conditions for the application of the XTT assay (7). We report herein the use of Alamar Blue, a recently developed fluorogenic compound for *in vitro* cytotoxicity measurement.

Correspondence to: Professor Michel Pagé, Department of Biochemistry, Faculty of Medicine, Université Laval, Québec G1K 7P4, Canada

Key words: fluorometric assay, cytotoxicity

Materials and methods

Cell lines. Cells used in this assay were mouse 3T3 and P388 cells obtained from the American Type Culture Collection (Bethesda, MA, USA); SKOV3, human ovarian carcinoma and SKVLB, human multidrug resistant ovarian carcinoma cells which were initially developed by Dr V. Ling in Toronto.

Cells were passaged once a week and cultured in RPM1-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% iron enriched bovine calf serum (Hyclone Laboratories, Logan, Utah) and 100 units each of penicillin and streptomycin. For resistant SKVLB cells, 100 ng/ml of vinblastin was added to the medium. Exponentially growing cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Alamar Blue is a fluorometric growth indicator obtained from Alamar Bio-Sciences Inc. (Sacramento, CA). This fluorescent indicator is supplied as a ready to use solution that may be stored in the dark at 4°C for long periods.

XTT used as a reference was purchased from Sigma Chemicals (St-Louis, MO, USA) and used as already described (7).

Measurements of fluorescence. Measurements of fluorescence were performed with the Millipore Cytofluor plate reader (Millipore-Waters, Mississauga, Ontario, Canada) and colorimetric assays were performed with the ELISA plate reader from Molecular Devices (Meno Park, CA).

Fluorescence spectra. Alamar Blue was reduced chemically by the addition of sodium borohydride and spectra of the

oxidized and reduced forms were measured using a RF-540 spectrofluorophotometer (Shimadzu Instruments).

Optimization of the fluorometric assay. To optimize the assay one variable was studied at a time. The Alamar Blue assay methodology was essentially the same as the one we have previously described for MTT and XTT (4) (7). Briefly, cells were inoculated into 96-well flat bottomed microplates (Becton Dickinson) in 200 µl of culture medium; various concentrations of Alamar Blue were added and cells were incubated at 37°C in the presence of 5% CO_2 for fluorescence development by living cells. After various periods, fluorescence was measured with the Cytofluor 2300 with an excitation at 530 nm and an emission at 590 nm. The Cytofluor System Software was used for data management.

Effect of time on fluorescence development. The reduction of Alamar Blue by cell dehydrogenases was studied with 12,500 SKOV3 cells by varying the incubation time from 0 to 8 hours using 10 microliters of Alamar Blue per well.

Effect of Alamar Blue concentration. The effect of Alamar Blue concentration was studied by incubating 12,500 SKOV3 cells/well for two hours in the presence of various concentrations of substrate at one sensitivity setting on the instrument.

Standard curves. Exponentially growing cells were counted and serially diluted in 100 µl of complete culture medium; 10 microliters of Alamar Blue was added per well and the microplate was incubated for two hours at 37°C before fluorescence measurement.

Cytotoxicity assay. Alamar Blue was compared to XTT to measure the cytotoxicity of daunorubicin *in vitro*. SKOV3 cells were plated at 2,500 cells per well in the presence of various concentrations (10-2,500 ng/ml) of daunorubicin (Rhone Poulenc Pharma, Montréal, Canada); in complete culture medium. Cells were incubated for four days at 37°C and the Alamar Blue assay was performed as described above and by the XTT method (7).

Results

Absorption spectra. Fig. 1 shows that the fluorescence of the oxidized and reduced forms of Alamar after excitation at 530 nm. The reduced form has a maximum emission at 590 nm while the fluorescence of the oxidized form is negligible.

Effect of incubation period. The effect of the incubation period of cells with Alamar Blue is given in Fig. 2. One may observe that in these assay conditions, an almost linear relationship was obtained for all concentrations used. However, when one microliter of Alamar was used per well, the fluorescence obtained was reduced about 3 fold. We found no significant difference in fluorescence development for volumes varying from 5 to 15 microliters per well.

Effect of concentration. Since Alamar Blue is already supplied in solution, the effect of substrate volume was studied on fluorescence development after two hours of incubation. In the assay conditions used, fluorescence development seems to reach a plateau with 15 microliters of Alamar and above (Fig. 3). The sensitivity setting is on a near logarithmic scale and the instrument is equiped with 10 sensitivity scales.

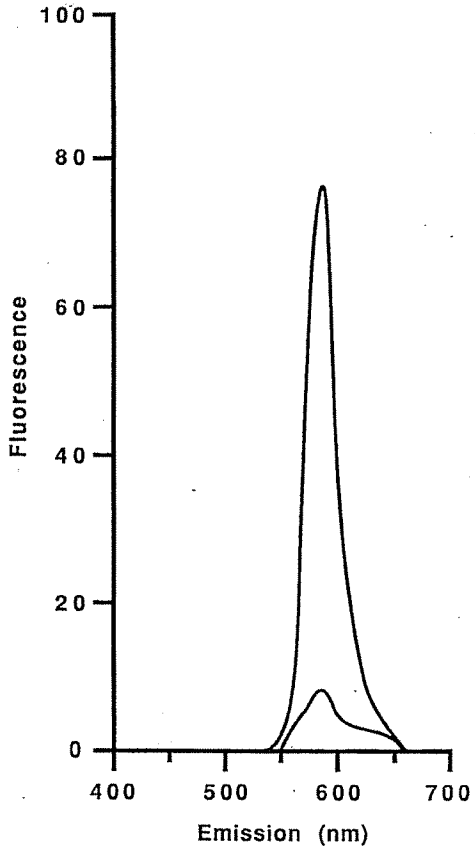

Figure 1. Absorption spectra of Alamar Blue in reduced and non-reduced forms. Alamar was reduced by addition of sodium borohydride.

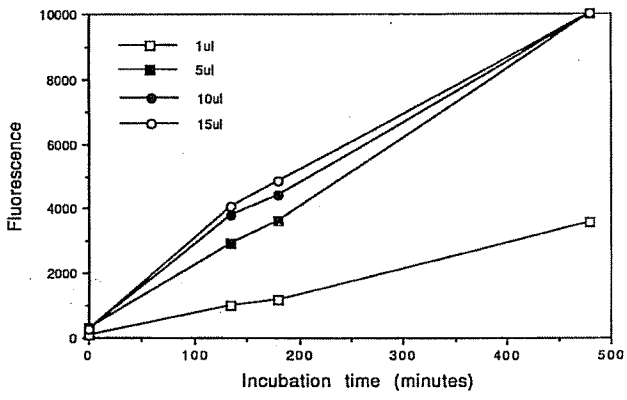

Figure 2. Time course for reduction of Alamar Blue (fluorescence development) by SKOV3 cells (12,500 cells/well), for different concentrations of Alamar Blue, 1 µl (□), 5 µl (■), 10 µl (●) and 15 µl (○) was added to the medium.

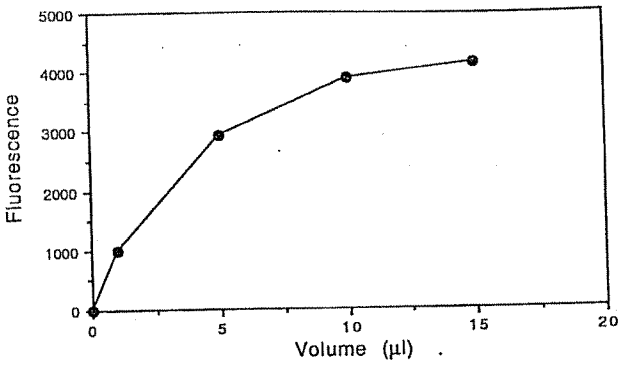

Figure 3. Optimisation assay using various volumes of Alamar Blue on SKOV3 cells (12,500 cells/well) for an incubation time of 2 hours.

Standard curves. Fig. 4 shows the relationship obtained between the fluorescence production and the cell concentration using four different cell lines. As we have already shown for other tests related to the activity of cellular dehydrogenases, a standard curve must be formed for each cell line due to intrinsic differences in the dehydrogenase content from one cell line to the other. We found a lower limit of detection of about 200 cells per well for most of the cell lines tested.

Cytotoxicity assay. Alamar Blue was compared to XTT to measure the cytotoxicity of daunorubicin *in vitro*; Fig. 5 shows that both assays are comparable in terms of IC_{50} which were 202 nM for XTT and 193 nM for Alamar Blue.

Discussion

Based on the results reported above, the optimal conditions for cell measurement using Alamar Blue could be summarized as follows: after proper experimental conditions, 10 microliters of Alamar Blue is added per well and incubated for two hours at 37°C. Fluorescence may then be read on the Millipore Cytofluor plate reader with an excitation at 530 nm and an emission at 590 nm. The optimal incubation period may vary from one cell line to the other but we found that for routine analysis with many cell lines, these conditions are suitable.

Results reported above show the advantages of this new fluorogenic indicator for cell measurement. Alamar blue yields a product which is soluble in culture medium.

Fig. 1 also shows that the background level with non reduced Alamar Blue is negligible. We found no interference with growth medium, however serum may cause some quenching. It is then recommended to use the same serum concentration in controls. The method may be used efficiently for measuring anchorage dependent or independent cell growth.

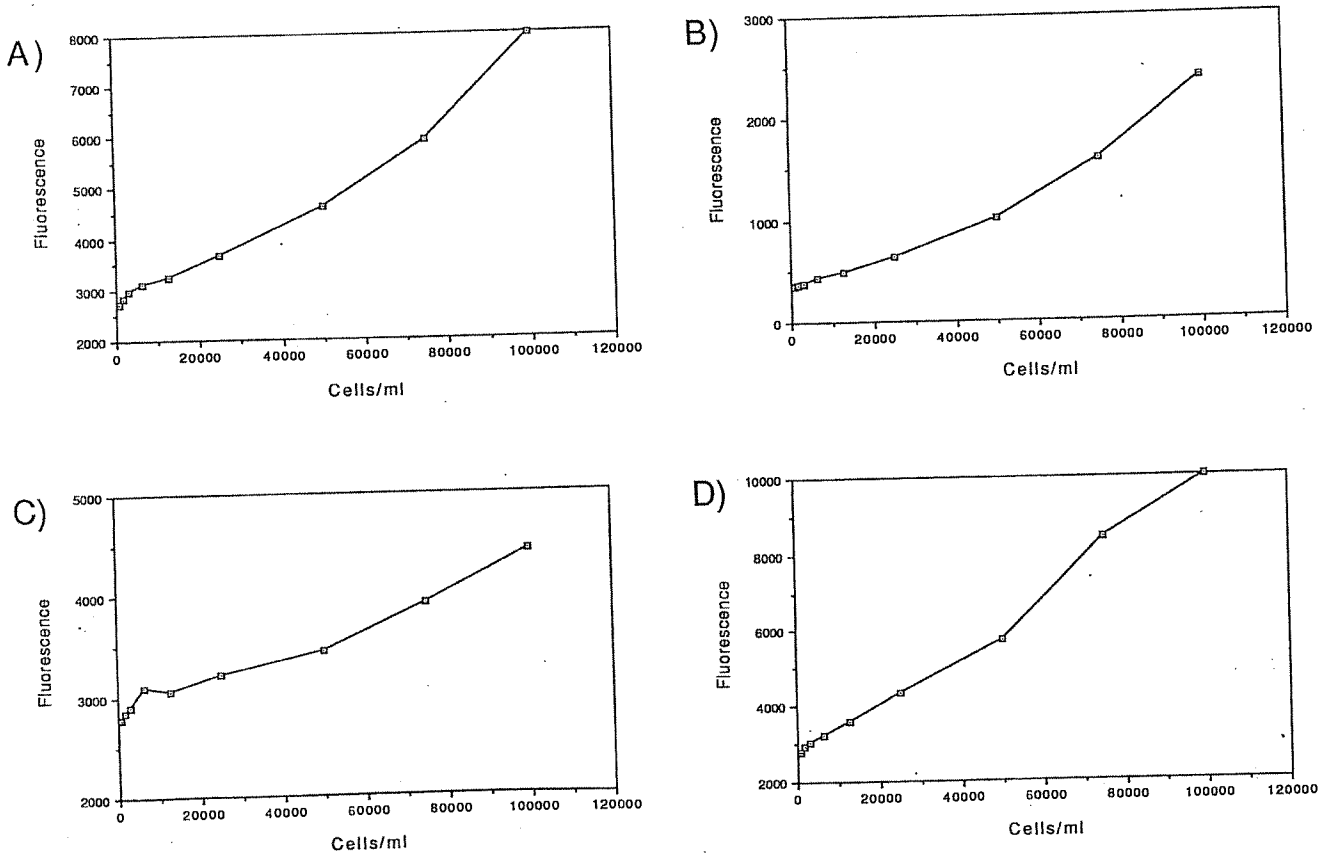

Figure 4. Standard curves for (A) SKVLB, (B) SKOV3, (C) P388 and (D) 3T3; incubation time 2 hours with 10 µl/well of Alamar Blue. Various concentrations of cells (serially diluted) were seeded in 96-well plates and Alamar Blue was added immediately.

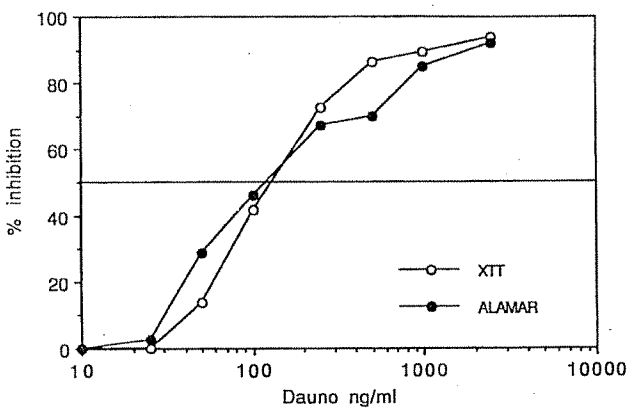

Figure 5. Cytotoxicity of daunorubicin on SKOV3 cells (12,500 cells/well). Cells were treated with various daunorubicin concentrations. On day 4 of treatment, the living cell population was measured by XTT or by the addition of 10 µl/well of Alamar Blue. Fluorescence was measured after an incubation of 2 hours.

This product could efficiently replace formazan salts or other cytotoxicity measurement techniques thus avoiding potentially hazardous solvents and long incubation periods for clinical evaluation. This method allowed a lower limit of detection of living cells and was used successfully in an adjunct project for measuring the activity of growth factors.

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EXHIBIT C

QUANTITATIVE ANALYSIS OF DOSE–EFFECT RELATIONSHIPS: THE COMBINED EFFECTS OF MULTIPLE DRUGS OR ENZYME INHIBITORS

TING-CHAO CHOU* and PAUL TALALAY†

*Laboratory of Pharmacology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, and †Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

INTRODUCTION

The quantitative relationship between the dose or concentration of a given ligand and its effect is a characteristic and important descriptor of many biological systems varying in complexity from isolated enzymes (or binding proteins) to intact animals. This relationship has been analyzed in considerable detail for reversible inhibitors of enzymes. Such analyses have made assumptions on the mechanism of inhibition (competitive, noncompetitive, uncompetitive), and on the mechanism of the reaction for multi-substrate enzymes (sequential or ping-pong), and have required knowledge of kinetic constants (1–4). More recently, it has been possible to describe the behavior of such enzyme inhibitors by simple generalized equations that are independent of inhibitor or reaction mechanisms and do not require knowledge of conventional kinetic constants (i.e. K_m, K_i, V_{max}) (5–8).

Our understanding of dose–effect relationships in pharmacological systems has not advanced to the same level as those of enzyme systems. Many types of mathematical transformations have been proposed to linearize dose-effect plots, based on statistical or empirical assumptions, e.g. probit (9, 10), logit (11) or power-law functions (12). Although these methods often provide adequate linearizations of plots, the slopes and intercepts of such graphs are usually devoid of any fundamental meaning.

THE MEDIAN EFFECT PRINCIPLE

We demonstrate here the application of a single and generalized method for analyzing dose–effect relationships in enzymatic, cellular and whole animal systems. We also examine the problem of quantitating the effects of multiple inhibitors on such systems and provide definitions of summation of effects, and consequently of synergism and antagonism.

Since the proposed method of analysis is derived from generalized mass action considerations, we caution the reader that the analysis of dose–effect

data is concerned with basic mass-action characteristics rather than with proof of specific mechanisms. Nevertheless, it is convenient and intuitively attractive to analyze and normalize all types of dose–response results by a uniform method which is based on sound fundamental considerations that have physicochemical and biochemical validity in simpler systems. Our analysis is based on the median effect principle of the mass action law (5–8), and has already been shown to be simple to apply and useful in the analysis of complex biological systems (13).

The Median Effect Equation

The median effect equation (6, 8) states that:

$$f_a/f_u = (D/D_m)^m \qquad (1)$$

where D is the dose, f_a and f_u are the fractions of the system affected and unaffected, respectively, by the dose D, D_m is the dose required to produce the median effect (analogous to the more familiar IC_{50}, ED_{50}, or LD_{50} values), and m is a Hill-type coefficient signifying the sigmoidicity of the dose–effect curve, i.e., m = 1 for hyperbolic (first order or Michaelis–Menten) systems. Since by definition, $f_a + f_u = 1$, several useful alternative forms of equation 1 are:

$$f_a/(1 - f_a) = [(f_a)^{-1} - 1]^{-1} = [(f_u)^{-1} - 1)] = (D/D_m)^m$$

$$f_a = 1/[1 + (D_m/D)]^m$$

$$D = D_m[f_a/(1 - f_a)]^{1/m}$$

The median effect equation describes the behavior of many biological systems. It is, in fact, a generalized form of the enzyme kinetic relations of Michaelis–Menten (14) and Hill (15), the physical adsorption isotherm of Langmuir (16), the pH-ionization equation of Henderson and Hasselbalch (17), the equilibrium binding equation of Scatchard (18), and the pharmacological drug–receptor interaction (19). Furthermore, the median effect equation is directly applicable not only to primary ligands such as substrates, agonists, and activators, but also to secondary ligands such as inhibitors, antagonists, or environmental factors (5, 6).

When applied to the analysis of the inhibition of enzyme systems, the median effect equation can be used without knowledge of conventional kinetic constants (i.e. K_m, V_{max} or K_i) and irrespective of the mechanism of inhibition (i.e. competitive, noncompetitive or uncompetitive). Furthermore, it is valid for multisubstrate reactions irrespective of mechanism (sequential or ping-pong) (5–8).

The Median Effect Plot

The median effect equation (equation 1) may be linearized by taking the logarithms of both sides, i.e.

$$\log (f_a/f_u) = m \log (D) - m \log (D_m)$$

or $$\log [(f_a)^{-1} - 1]^{-1} = m \log (D) - m \log (D_m)$$

or $$\log [(f_u)^{-1} - 1] = m \log (D) - m \log (D_m)$$

The median effect plot (Fig. 1) of $y = \log (f_a/f_u)$ or its equivalents with respect to $x = \log (D)$ is a general and simple method (13, 30) for determining pharmacological median doses for lethality (LD_{50}), toxicity (TD_{50}), effect of agonist drugs (ED_{50}), and effect of antagonist drugs (IC_{50}). Thus, the median-effect principle of the mass-action law encompasses a wide range of applications. The plot gives the slope, m, and the intercept of the dose-effect plot with the median-effect axis [i.e. when $f_a = f_u$, $f_a/f_u = 1$ and hence $y = \log (f_a/f_u) = 0$] which gives $\log (D_m)$ and consequently the D_m value. Any cause-consequence relationship that gives a straight line for this plot will provide the two basic parameters, m and D_m, and thus, an apparent equation that describes such a system. The linearity of the median-effect plot (as determined from linear regression coefficients) determines the applicability of the present method.

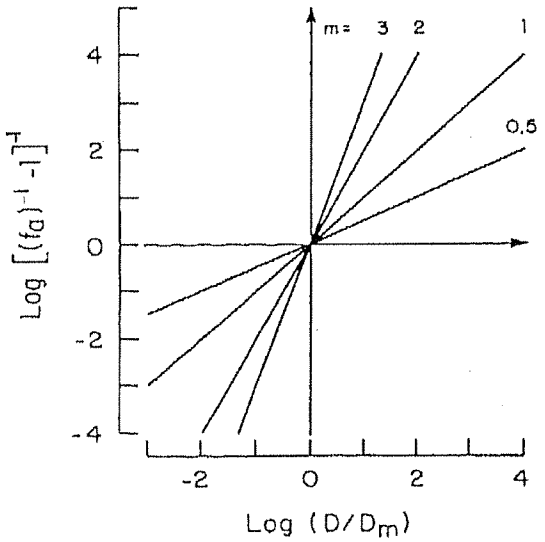

FIG. 1. The median-effect plot at different slopes corresponding to m values of 0.5, 1, 2 and 3. The plot is based on the median-effect equation (equation 1) in which the dose (D) has been normalized by taking the ratio to the median-effect dose (D_m). Note that the ordinate $\log [(f_a)^{-1} - 1]^{-1}$ is identical to $\log [f_u)^{-1} - 1]$ or $\log (f_a/f_u)$.

Relation of the Median-Effect Equation to Michaelis–Menten and Hill Equations

In the special case, when m = 1, equation 1 becomes $f_a = [1 + (D_m/D]^{-1}$ which has the same form as the Michaelis–Menten equation (14), $v/V_{max} = [1 + (K_m/S)]^{-1}$. In addition, when the effector ligand is an environmental factor such as an inhibitor, the equation, $f_a = [1 + (D_m/D)]^{-1}$, is valid not only for a single substrate reaction (Michaelis–Menten equation) but also for multiple substrate reactions; the fractional effect is expressed with respect to the control velocity rather than to the maximal velocity (6). Furthermore, f_a in equation 1 is simple to obtain, whereas the determination of V_{max} in the Michaelis–Menten (or Hill) equations requires approximation or extrapolation (6, 7). The logarithmic form of equation 1 describes the Hill equation.

The Utility of the Median Effect Principle

The median-effect equation has been used to obtain accurate values of IC_{50}, ED_{50}, LD_{50}, or the relative potencies of drugs or inhibitors in enzyme systems (6–8, 21–26), in cellular systems (20, 27, 28) and in animal systems (13, 29–32). An alternative form of the median-effect equation (5) has been used for calculating the dissociation constant (K_i or K_a) of ligands for pharmacological receptors (33–35). It has also permitted the analysis of chemical carcinogenesis data and has predicted especially accurately tumor incidence at low dose carcinogen exposure (30, 31). By using the median-effect principle, the general equation for describing a standard radioimmunoassay or ligand displacement curve has been derived recently by Smith (36). It has also been used to show that there is marked synergism among chemotherapeutic agents in the treatment of hormone-responsive experimental mammary carcinomas (32). In recent preliminary reports (13, 37), we have shown that, in conjunction with the multiple drug effect equations (see below), the median-effect plot forms the basis for the quantitation of synergism, summation and antagonism of drug effects.

ANALYSIS OF MULTIPLE DRUG EFFECTS

An Overview

Over the past decades, numerous authors have claimed synergism, summation or antagonism of the effects of multiple drugs. However, there is still no consensus as to the meanings of these terms. For instance, in a review, Goldin and Mantel in 1957 (38) listed seven different definitions of these terms. Confusion and ambiguity persist (39) despite increasing use of multiple drugs in experimentation and in therapy. This emphasizes the lack of a

theoretical basis that would permit rigorous and quantitative assessment of the effects of drug combinations.

Attempts to interpret the effect of multiple drugs have been documented for more than a century (39). Since the introduction of the isobol concept by Loewe in 1928 (40, 41) and the fractional product concept (see Appendix) by Webb in 1963 (42), the theoretical and practical aspects of the problem have been the subject of many reviews (38, 43–51). Some authors have discussed the possible mechanisms that may lead to synergism, and others have emphasized methods of data analysis. The kinetic approach was used earlier by some investigators (4, 42, 52–58), but the formulations were frequently too complex to be of practical usefulness or were restricted to individual situations. Although not specifically stated, some formulations are limited to two inhibitors; others are valid only for first order (Michaelis–Menten type) systems but not for higher order (Hill type) systems, and still others are valid only for mutually nonexclusive inhibitors but not for mutually exclusive inhibitors.

The present authors, therefore, have undertaken a kinetic approach to analyze the problem. An unambiguous definition of summation is a prerequisite for any meaningful conclusions with respect to synergism and antagonism. Ironically, two prevalent concepts for calculating summation i.e., the isobol and the fractional-product method, are shown to conform to two opposite situations. The former concept is valid for drugs whose effects are mutually exclusive, and the latter is valid for mutually nonexclusive drugs (13, 49), and thus these methods cannot be used indiscriminately (see Appendix). In this paper, we provide the equations for both situations and show that they are merely special cases of the general equations described recently (59). We also propose a general diagnostic plot to determine the applicability of experimental data, to distinguish mutually exclusive from nonexclusive drugs, and to obtain parameters that can be directly used for the analysis of summation, synergism or antagonism.

Requirements for Analyzing Multiple Drug Effects

The following information is essential for analyzing multiple drug effects and for quantitating synergism, summation and antagonism of multiple drugs.

1. A quantitative definition of summation is required since synergism implies more than summation and antagonism less than summation of effects.

2. Dose–effect relationships for drug 1, drug 2 and their mixture (at a known ratio of drug 1 to drug 2) are required.

a. Measurements made with single doses of drug 1, drug 2 and their mixture can never alone determine synergism since the sigmoidicity of dose–effect

curves and the exclusivity of drug effects cannot be determined from such measurements.

b. The dose–effect relationships should follow the basic mass-action principle relatively well (e.g. median–effect plots with correlation coefficients for the regression lines greater than 0.9).

c. Determination of the sigmoidicity of dose–effect curves and the exclusivity of effects of multiple drugs is necessary. The slope of the median-effect plot gives a quantitative estimation of sigmoidicity. When $m = 1$, the dose-effect curve is hyperbolic; when $m \neq 1$, the dose–effect curve is sigmoidal, and the greater the m value, the greater its sigmoidicity; $m < 1$ is a relatively rare case which in allosteric systems indicates negative cooperativity of drug binding at the receptor sites. When the dose–effect relationships of drug 1, drug 2 and their mixture are all parallel in the median-effect plot, the effects of drug 1 and drug 2 are mutually exclusive (59). If the plots of drugs 1 and 2 are parallel but the plot of their mixture is concave upward with a tendency to intersect the plot of the more potent of the two drugs, their effects are mutually nonexclusive (59). If the plots for drugs 1 and 2 and their mixture are not parallel to each other, exclusivity of effects cannot be established. Alternatively, exclusivity of effects may not be ascertained because of a limited number of data points or limited dose range. In these cases, the data may be analyzed for the "combination index" (see below) on the basis of both mutually exclusive and mutually nonexclusive assumptions. Note that exclusivity may occur at a receptor site, at a point in a metabolic pathway, or in more complex systems, depending on the endpoint of the measurements.

Equations for the Effects of Multiple Drugs

A systematic analysis in enzyme kinetic systems using the basic principles of the mass action law has led to the derivation of generalized equations for multiple inhibitors or drugs (8, 59).

1. *For two mutually exclusive drugs that obey first order conditions.* If two drugs (e.g., inhibitors D_1 and D_2) have effects that are mutually exclusive, then the summation of combined effects $(f_a)_{1,2}$, in first-order systems (i.e., each drug follows a hyperbolic dose–effect curve) can be calculated from (59):

$$\frac{(f_a)_{1,2}}{(f_u)_{1,2}} = \frac{(f_a)_1}{(f_u)_1} + \frac{(f_a)_2}{(f_u)_2}$$

$$= \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} \qquad (2)$$

where f_a is the fraction affected and f_u is the fraction unaffected, and ED_{50} is the concentration of the drug that is required to produce a 50% effect. Note that $f_a + f_u = 1$ or $f_a = 1 - f_u$.

2. *For two mutually nonexclusive drugs that obey first order conditions.* If the effects of two drugs (D_1 and D_2) are mutually non-exclusive (i.e., they have different modes of action or act independently) the summation of combined effects, $(f_a)_{1,2}$, in a first-order system is (59):

$$\frac{(f_a)_{1,2}}{(f_u)_{1,2}} = \frac{(f_a)_1}{(f_u)_1} + \frac{(f_a)_2}{(f_u)_2} + \frac{(f_a)_1\,(f_a)_2}{(f_u)_1\,(f_u)_2}$$

$$= \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} + \frac{(D)_1\,(D)_2}{(ED_{50})_1\,(ED_{50})_2} \qquad (3)$$

Similar relationships apply to situations involving more than two inhibitors, for which generalized equations are given in ref. 59. In enzyme systems, equations 2 and 3 express summation of inhibitory effects, irrespective of the number of substrates, the type or mode of reversible inhibition (competitive, noncompetitive or uncompetitive) or the kinetic mechanisms (sequential or ping-pong) of the reaction under consideration. The simplicity of the above equations (in which all specific kinetic constants, substrate concentration factors, and V_{max} have been cancelled out during derivation) suggests their general applicability (5, 6). This is in contrast to the mechanism-specific reactions (3, 5) for which the equations are far more complex. In more organized cellular or animal systems, the dose–effect relationships of drugs or inhibitors are frequently sigmoidal rather than hyperbolic.

3. *For two mutually exclusive drugs that obey higher order conditions.* The above concepts have been extended to higher-order (Hill-type) systems in which each drug has a sigmoidal dose–effect curve (i.e., has more than one binding site or exhibits positive or negative cooperativity). If the effects of such drugs are mutually exclusive:

$$\left[\frac{(f_a)_{1,2}}{(f_u)_{1,2}}\right]^{\frac{1}{m}} = \left[\frac{(f_a)_1}{(f_u)_1}\right]^{\frac{1}{m}} + \left[\frac{(f_a)_2}{(f_u)_2}\right]^{\frac{1}{m}} = \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} \qquad (4)$$

where m is a Hill-type coefficient which denotes the sigmoidicity of the dose–effect curve.

4. *For two mutually nonexclusive drugs that obey higher order conditions.* If the effects of two drugs (D_1 and D_2) are mutually nonexclusive and if each drug and their combination follow a sigmoidal dose–effect relationship with m^{th} order kinetics, then this relationship becomes (59):

$$\left[\frac{(f_a)_{1,2}}{(f_u)_{1,2}}\right]^{\frac{1}{m}} = \left[\frac{(f_a)_1}{(f_u)_1}\right]^{\frac{1}{m}} + \left[\frac{(f_a)_2}{(f_u)_2}\right]^{\frac{1}{m}} + \left[\frac{(f_a)_1\,(f_a)_2}{(f_u)_1\,(f_u)_2}\right]^{\frac{1}{m}}$$

$$= \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} + \frac{(D)_1\,(D)_2}{(ED_{50})_1\,(ED_{50})_2} \qquad (5)$$

In the special case where $(f_a)_{1,2} = (f_u)_{1,2} = 0.5$, equations 2 and 4 become:

$$\frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} = 1 \qquad (6)$$

which describes the ED_{50} isobologram.

Similarly, equations 3 and 5 become:

$$\frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} + \frac{(D)_1\,(D)_2}{(ED_{50})_1\,(ED_{50})_2} = 1 \qquad (7)$$

which does not describe an isobologram, because of the additional term on the left.

In the Appendix it is shown that equation 3 or 7 can be readily used for deriving the fractional product equation of Webb (42), and equation 4 can be used for deriving the generalized isobologram equation for any desired f_a value. Thus, for the isobologram at any fractional effect f_a = x per cent, the generalized equation is:

$$\frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = 1 \qquad (8)$$

The limitations of the fractional product concept and the isobologram method are detailed in the Appendix.

5. *Quantitation of synergism, summation and antagonism.* When experimental results are entered into equations 2–5, if the sum of the two terms in equation 2 or 4, or the sum of the three terms in equation 3 or 5 is greater than, equal to, or smaller than 1, it may be inferred that antagonism, summation or synergism of effects, respectively, has been observed. Therefore, from equations 2–5, if the combined observed effect is greater than the calculated additive effect, $(f_a)_{1,2}$, synergism is indicated; if it is smaller, antagonism is indicated.

It is, however, convenient to designate a "combination index" (CI) for quantifying synergism, summation, and antagonism, as follows:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \tag{9}$$

for mutually exclusive drugs, and

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1\,(D)_2}{(D_x)_1\,(D)_x)_2} \tag{10}$$

for mutually nonexclusive drugs.

For mutually exclusive or nonexclusive drugs,

when $CI < 1$, synergism is indicated.

$CI = 1$, summation is indicated.

$CI > 1$, antagonism is indicated.

To determine synergism, summation and antagonism at any effect level (i.e., for any f_a value), the procedure involves three steps: i) Construct the median-effect plot (Eqn. 1) which determines m and D_m values for drug 1, drug 2 and their combination; ii) for a given degree of effect (i.e., a given f_a value representing x per cent affected), calculate the corresponding doses [i.e., $(D_x)_1$, $(D_x)_2$ and $(D_x)_{1,2}$] by using the alternative form of equation 1, $D_x = D_m [f_a/(1 - f_a)]^{1/m}$; iii) calculate the combination index (CI) by using equations 9 or 10, where $(D_x)_1$ and $(D_x)_2$ are from step (ii), and $(D_x)_{1,2}$ [also from step (ii)] can be dissected into $(D)_1$ and $(D)_2$ by their known ratio, P/Q. Thus, $(D)_1 = (D_x)_{1,2} \times P/(P + Q)$ and $(D)_2 = (D_x)_{1,2} \times Q/(P + Q)$. CI values that are smaller than, equal to, or greater than 1, represent synergism, summation and antagonism, respectively.

To facilitate the calculation, a computer program written in BASIC for automatic graphing of CI with respect to f_a has been developed. Samples of this computer simulation are shown in the examples to be given later. A sample calculation of CI without using a computer is also given in Example 1.

APPLICATIONS OF THE MEDIAN EFFECT EQUATION AND PLOTS TO THE ANALYSIS OF MULTIPLE DRUGS OR INHIBITORS

Example I. Inhibition of Alcohol Dehydrogenase by Two Mutually Exclusive Inhibitors

Yonetani and Theorell (55) have reported the inhibition of horse liver alcohol dehydrogenase by two inhibitors (I_1 = ADP-ribose and I_2 = ADP) both of which are competitive with respect to NAD. Velocity measurements in the presence of a range of concentrations of the two inhibitors (alone and in combination) and control velocities were retrieved from the original plot, and tabulated in ref. 59. The results are most conveniently expressed as fractional velocities (f_v) which are the ratios of the inhibited velocities to the control velocities, and therefore correspond to the fraction of the process unaffected (f_u). The fractional velocities in the presence of ADP-ribose (95–375 μM), ADP (0.5–2.5 μM), and a combination of ADP-ribose and ADP at a constant molar ratio of 190:1, have been plotted as $\log[(f_v)^{-1} - 1]$ with respect to $\log(I)$ (Fig. 2). For ADP-ribose, $m = 0.968$, $I_{50} = 156.1$ μM with a regression coefficient of $r = 0.9988$. For ADP, $m = 1.043$, $I_{50} = 1.657$ μM and $r = 0.9996$. For ADP-ribose and ADP in combination (molar ratio 190:1), $m_{1,2} = 1.004$, $(I_{50})_{1,2} = 107.0$ μM and $r = 0.9997$. It is clear that both inhibitors follow first-order kinetics (i.e., $m \approx 1$) and that ADP-ribose and ADP are mutually exclusive inhibitors (i.e., the

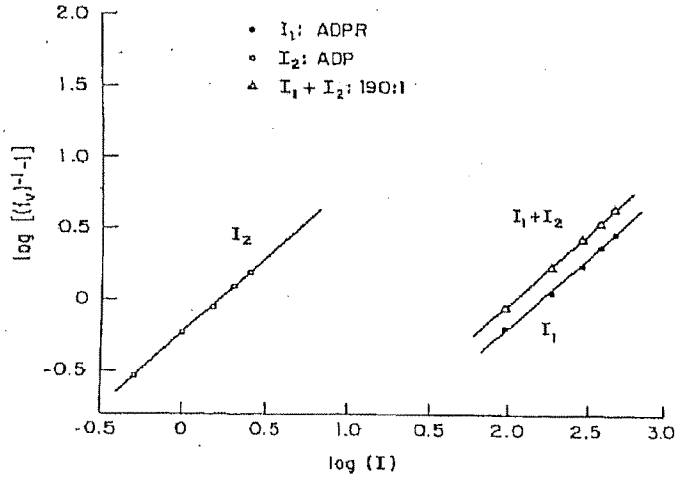

FIG. 2. Median-effect plots of the experimental data of Yonetani and Theorell (55) for the inhibition of horse liver alcohol dehydrogenase by two mutually exclusive inhibitors. I_1 is ADP-ribose (ADPR), I_2 is ADP, and $I_1 + I_2$ is a mixture of ADP-ribose and ADP in a molar ratio of 190:1. The abscissa represents $\log(I)_1$ (●), $\log(I)_2$ (○), or $\log[(I)_1 + (I)_2]$(190:1; △). In this case it is convenient to use the terms fractional velocity (f_v) which is the ratio of the inhibited to the control velocity and therefore corresponds to the fraction that is unaffected (f_u). [from Chou and Talalay (59)].

plot for the combination of inhibitors parallels the plots for each of the component inhibitors). These conclusions are in agreement with the interpretations obtained by Yonetani and Theorell (55) and Chou and Talalay (59) using different methods. For the present analysis, knowledge of kinetic constants and type of inhibition is not required. The plots show excellent agreement between theory and experiment.

With this knowledge of the m and I_{50} values for each inhibitor and the combination at a constant molar ratio, it is possible to calculate the inhibitor combination index (CI) for a series of values of f_a (Fig. 3). The CI values are close to 1 over the entire range of f_a values, suggesting strongly that the inhibitory effects of ADP-ribose and ADP are additive.

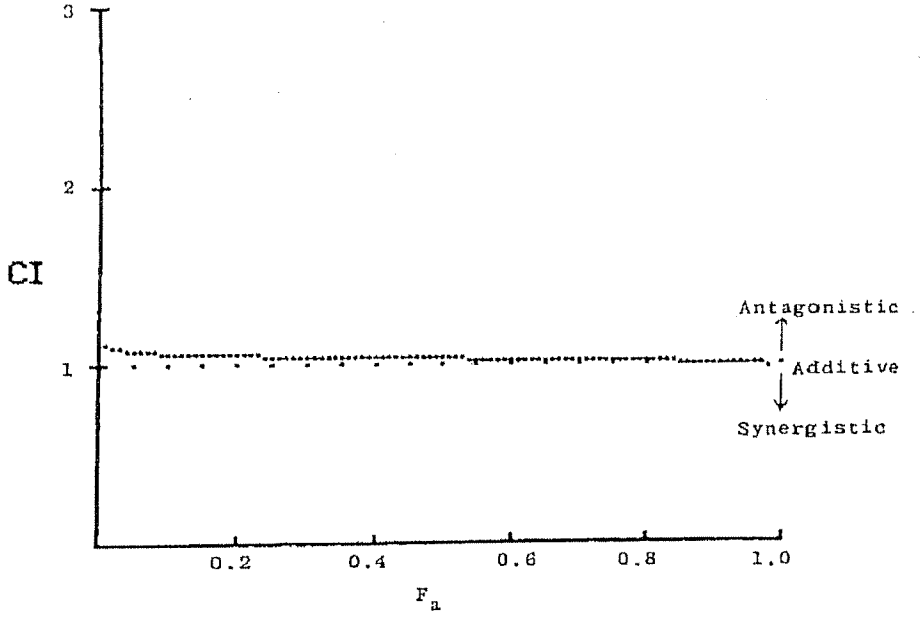

FIG. 3. Computer-generated graphical presentation of the combination index (CI) with respect to fraction affected (f_a) for the additive inhibition by ADP-ribose and ADP (molar ratio of 190:1) of horse liver alcohol dehydrogenase. The plot is based on equation 9 (mutually exclusive) as described in the section entitled "Quantitation of Synergism, Summation and Antagonism." CI is the combination index which is equal to $(D)_1/(D_x)_1 + (D)_2/(D_x)_2$ (see text for sample calculation). $CI < 1$, $= 1$ and > 1 represent synergistic, additive and antagonistic effects, respectively. Although plots of CI with respect to f_a can be obtained by step-by-step calculations, it is much more convenient to use computer simulation. The parameters were obtained as described in Fig. 2, by the use of linear regression analysis or computer simulation.

We now give a sample calculation of the combination index (CI) for an arbitrarily selected value of $f_a = 0.9$:

From equation 1, $D_x = D_m [f_a/(1 - f_a)]^{1/m}$.

Since I_1 is ADP-ribose and I_2 is ADP,

then $(D_{90})_1 = 156.1\ \mu M\ [0.9/(1 - 0.9)]^{1/0.968} = 1511\ \mu M$

$(D_{90})_2 = 1.657\ \mu M\ [0.9/(1 - 0.9)]^{1/1.043} = 13.62\ \mu M$

$(D_{90})_{1,2} = 107.0\ \mu M\ [0.9/(1 - 0.9)]^{1/1.004} = 954.6\ \mu M$

Since in the mixture $I_1 : I_2 = 190:1$,
then, $(D_{90})_{1,2}$ can be dissected into:

$$(D)_1 = 954.6 \times [190/(190 + 1)] = 949.6\ \mu M$$
$$(D)_2 = 954.6 \times [1/(190 + 1)] = 4.998\ \mu M$$

$$\text{therefore, } (CI)_{90} = \frac{949.6\ \mu M}{1511\ \mu M} + \frac{4.998\ \mu M}{13.62\ \mu M} = 0.9955.$$

Since value of $(CI)_{90}$ is close to 1, an additive effect of ADP-ribose and ADP at $f_a = 0.9$ is indicated.

A computer program for automated calculation of m, D_m, D_x, r, and CI at different f_a values has been developed.

Example 2. Inhibition of Alcohol Dehydrogenase by Two Mutually Non-Exclusive Inhibitors

Yonetani and Theorell (55) also studied the inhibition of horse liver alcohol dehydrogenase by the two competitive, mutually nonexclusive inhibitors: *o*-phenanthroline (I_1) and ADP (I_2). The fractional velocity (f_v) values retrieved from the original plot are given in ref. 59, and are presented in the form of a median-effect plot, i.e., log $[(f_v)^{-1} - 1]$ with respect to log (I) (Fig. 4). *o*-Phenanthroline gives m = 1.303, $I_{50} = 36.81\ \mu M$ and r = 0.9982, and ADP gives m = 1.187, $I_{50} = 1.656\ \mu M$ and r = 0.9842. These data again show that both inhibitors follow first order kinetics (i.e., m ≈ 1). However, when the data for

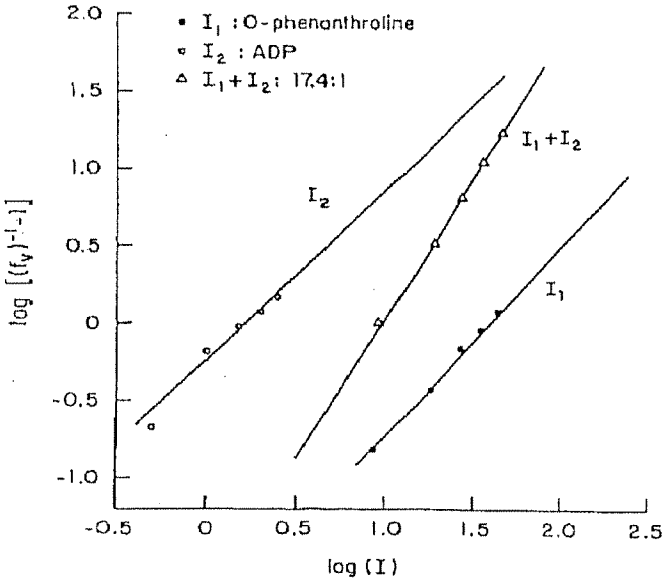

FIG. 4. The median-effect plot of experimental data of Yonetani and Theorell (55) for the inhibition of horse liver alcohol dehydrogenase by two mutually nonexclusive inhibitors. I_1 is *o*-phenanthroline, I_2 is ADP, and $I_1 + I_2$ is a mixture of *o*-phenanthroline and ADP (molar ratio 17.4:1). The abscissa represents log $(I)_1$ (●), log $(I)_2$ (○), or log $[(I)_1 + (I)_2]$ (△) [from Chou and Talalay (59)].

the mixture of *o*-phenanthroline and ADP (constant molar ratio 17.4:1) are plotted in the same manner, a very different result is obtained: $m_{1,2} = 1.742$ (apparent), $(I_{50})_{1,2} = 9.116$ μM and $r = 0.9999$. The dramatic increase in the slope of the plot for the mixture (in comparison to each of its components), clearly indicates that *o*-phenanthroline and ADP are mutually nonexclusive inhibitors.

The combination indices at various f_a levels are given in Figure 5. The results indicate that there is a moderate antagonism at low f_a values and a marked synergism at high f_a values.

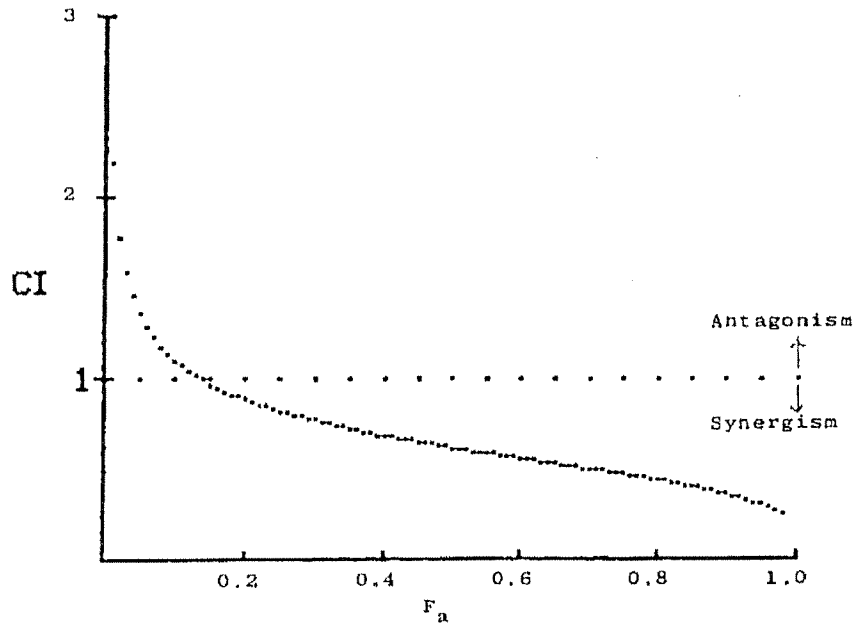

FIG. 5. Computer-generated graphical presentation of the combination index (CI) with respect to fraction affected (f_a) for the inhibition of horse liver alcohol dehydrogenase by a mixture of *o*-phenanthroline and ADP (molar ratio 17.4:1). The method of analysis is the same as that described in the legend to Fig. 3, except that equation 10 (mutually non-exclusive) is used.

Example 3. Inhibition of the Incorporation of Deoxyuridine into the DNA of L1210 Leukemia Cells by Methotrexate (MTX) and 1-β-D-Arabinofuranosylcytosine (ara-C)

Murine L1210 leukemia cells were incubated in the presence of a range of concentrations of MTX (0.1–6.4 μM), of ara-C (0.0782–5.0 μM), or a constant molar ratio mixture of MTX and ara-C (1:0.782), and the incorporation of deoxyuridine into DNA was then determined. The fractional inhibitions (f_a) of dUrd incorporation are shown in Table 1. Analysis of the results by the median effect plot (Fig. 6) gave the following parameters: for MTX, $m = 1.091$, $D_m = 2.554$ μM, $r = 0.9842$; for ara-C, $m = 1.0850$, $D_m = 0.06245$ μM, and

TABLE 1. INHIBITION OF [6-^{3}H]DEOXYURIDINE (dUrd) INCORPORATION INTO DNA IN L1210 LEUKEMIA CELLS BY METHOTREXATE (MTX) AND 1-β-D-ARABINOFURANOSYLCYTOSINE (ARA-C), ALONE AND IN COMBINATION

	Fractional inhibition (f_a) at [ara-C] of							
MTX μM	0	0.782 μM	0.156 μM	0.313 μM	0.625 μM	1.25 μM	2.5 μM	5.0 μM
0	0	0.582	0.715	0.860	0.926	0.955	0.980	0.993
0.1	0.0348	0.405						
0.2	ND*		0.587					
0.4	ND			0.775				
0.8	0.140				0.878			
1.6	0.415					0.943		
3.2	0.573						0.970	
6.4	0.755							ND

*Result not used because of large variation between duplicates.

L1210 murine leukemia cells (8×10^6 cells) were incubated in Eagle's basal medium (20) in the presence and absence of various concentrations of MTX and ara-C and their mixture (molar ratio, 1:0.782) at 37°C for 20 min and then incubated with 0.5 μM (1 μCi) of [6-^{3}H]dUrd, at 37°C for 30 min. Fractional inhibition (f_i or f_a) of [6-^{3}H]dUrd incorporation into perchloric acid-insoluble DNA fraction was then measured as previously described (20). All measurements were made in duplicate.

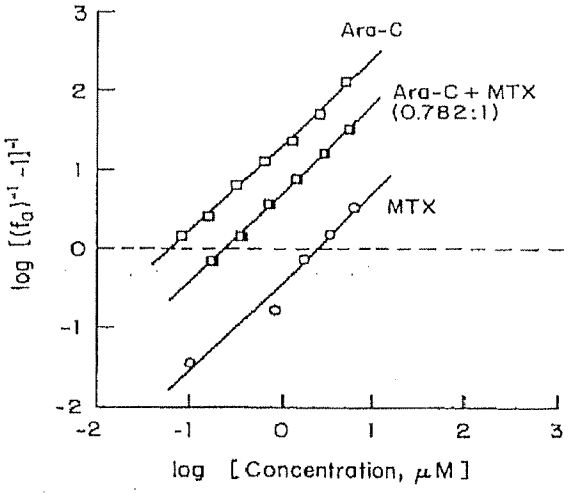

FIG. 6. Median-effect plot showing the inhibition of [6-^{3}H]dUrd incorporation into DNA of L1210 murine leukemia cells by methotrexate (MTX), (O); arabinofuranosylcytosine (ara-C), (×); or their mixture (1:0.782), (+). Data from Table 1 have been used.

r = 0.9995. For the combination of MTX and ara-C (1:0.782), the parameters were: m = 1.1296, D_m = 0.2496 μM, and r = 0.9995. The combination index (Fig. 7) shows a moderate antagonism between the two drugs at all values of fractional inhibition.

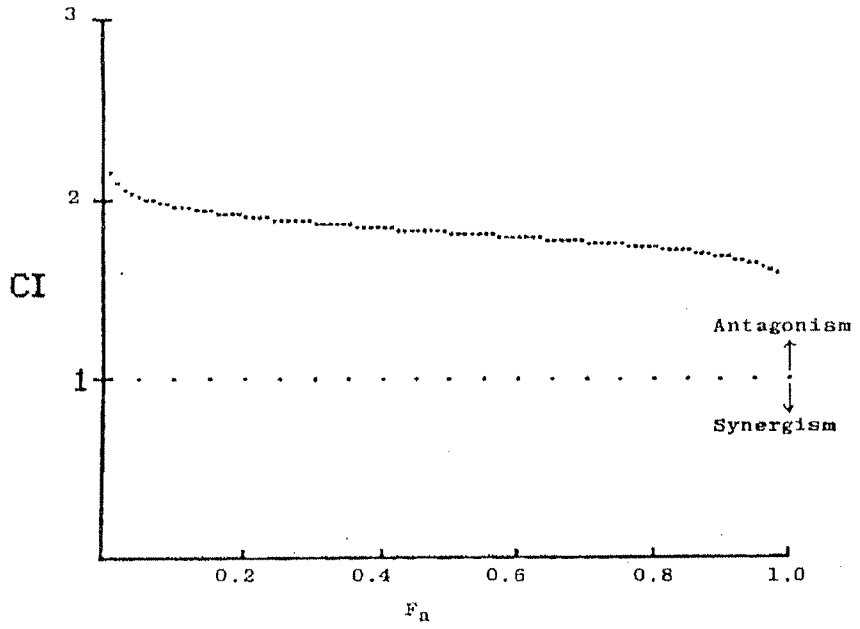

FIG. 7. Computer-generated graphical presentation of the drug combination index (CI) with respect to fraction affected (f_a) for the inhibitory effect of a mixture of methotrexate (MTX) and arabinofuranosylcytosine (ara-C) (molar ratio, 1:0.782) on the incorporation of [6-^{3}H]dUrd into DNA of L1210 murine leukemia cells. The data from Table 1 and the parameters obtained from Fig. 6 have been used for this plot on the assumption that the drugs act in a mutually exclusive manner (equation 9).

Example 4. Inhibition of the Incorporation of Deoxyuridine into the DNA of L1210 Leukemia Cells by Hydroxyurea (HU) and 5-Fluorouracil (5-FU)

Murine L1210 leukemia cells were incubated in the presence of a range of concentrations of hydroxyurea (50–3,200 μM), or 5-fluorouracil (4.0–256 μM), and of a constant molar ratio mixture of hydroxyurea and 5-fluorouracil (12.5:1), and the incorporation of deoxyuridine (dUrd) into DNA was then determined. The fractional inhibitions (f_a) of dUrd incorporation are shown in Table 2. Analysis of the results by the median effect plot (Fig. 8) gave the following parameters: for hydroxyurea, m = 1.196, D_m = 34.09 μM, and r = 0.9908; for 5-fluorouracil, m = 1.187, D_m = 8.039 μM, and r = 0.9978; and for the mixture of hydroxyurea and 5-fluorouracil (12.5:1), m = 1.407, D_m = 225.8 μM, and r = 0.9776. It is immediately apparent from Figure 8 that the effects of hydroxyurea and 5-fluorouracil are markedly antagonistic since the median-effect plot for the mixture lies to the right of both parent compounds. The degree of this antagonism falls as the level of inhibition increases, i.e. as f_a increases (Table 3). The reasons for this marked antagonism are obscure, but may be of practical importance.

TABLE 2. INHIBITION OF [6-^{3}H]DEOXYURIDINE INCORPORATION INTO DNA IN L1210 LEUKEMIA CELLS BY HYDROXYUREA (HU), AND 5-FLUOROURACIL (5-FU), ALONE AND IN COMBINATION

	Fractional inhibition (f_a) at [5-FU] of							
HU μM	0	4 μM	8 μM	16 μM	32 μM	64 μM	128 μM	256 μM
0	0	0.348	0.475	0.661	0.827	0.923	0.966	0.985
50	0.605	0.208						
100	0.741		0.168					
200	0.889			0.345				
400	0.962				0.747			
800	0.984					0.885		
1,600	0.990						0.957	
3,200	0.994							0.977

L1210 murine leukemia cells (4.5×10^6) were incubated as described in the legend to Table 1, except that the incubation period with drugs prior to the addition of [6-^{3}H]dUrd was 40 min. The results are analyzed in Fig. 8 and Table 3.

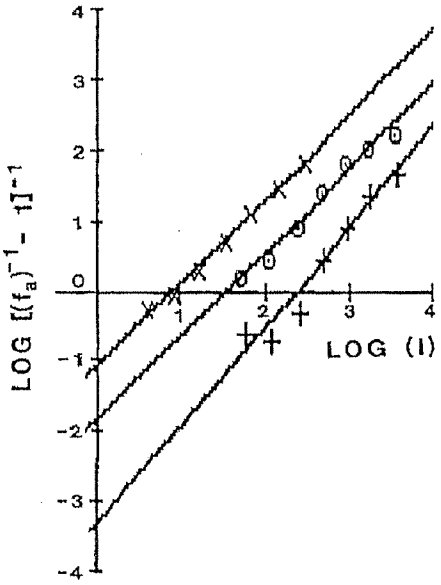

FIG. 8. Computer-generated median effect plot showing the inhibition of the incorporation of [6-^{3}H]dUrd into DNA of L1210 murine leukemia cells by hydroxyurea (HU), (⊡); 5-fluorouracil (5-FU), (×), or their mixture (12.5:1), (+). The data given in Table 2 have been used. The parameters (m, D_m and r) can be obtained automatically.

Example 5. The Lethal Effects of Two Insecticides on Houseflies

Nearly 50 years ago Le Pelley and Sullivan (60) reported very careful dose–effect data for the lethality of rotenone, pyrethrins, and a mixture of these insecticides on houseflies. Adult houseflies were sprayed with alcoholic solutions of rotenone, pyrethrins, and a mixture of the two insecticides in a

TABLE 3. CALCULATED VALUES FOR THE COMBINATION INDEX AS A FUNCTION OF FRACTIONAL INHIBITION (F_a) OF THE INCORPORATION OF [6-^{3}H]DEOXYURIDINE INTO DNA OF L1210 LEUKEMIA CELLS BY A MIXTURE OF HYDROXYUREA AND 5-FLUOROURACIL (MOLAR RATIO 12.5:1)

Fractional inhibition (f_a)	Combination index (CI)	Diagnosis of combined effect
0.05	11.95	Antagonism
0.10	10.86	Antagonism
0.20	9.80	Antagonism
0.30	9.15	Antagonism
0.40	8.65	Antagonism
0.50	8.21	Antagonism
0.60	7.80	Antagonism
0.70	7.38	Antagonism
0.80	6.89	Antagonism
0.90	6.21	Antagonism
0.95	5.61	Antagonism

The combination index was calculated on the assumption that the two drugs are mutually exclusive (Eqn. 9). The combination index was generated by computer on the basis of parameters obtained from the median effect plot (Fig. 8).

ratio by weight of 1:5. One thousand flies were used for each dose. The data are of historical interest since four different laboratories have attempted to answer the question whether there is synergism among these insecticides. The numerical results were retrieved by Finney (9) from the diagrams contained in the original paper (60).

These quantal data were equally suitable for analysis by the median effect principles as were the earlier examples in which graded responses were analyzed. We have recently provided a preliminary analysis of these results (13). The parameters of the median effect equation are shown in Table 4.

The dose–effect relationships for rotenone, pyrethrins and their mixture are clearly sigmoidal (Fig. 9A) with slopes (m values) ranging from 2.52 to 2.75 (Fig. 9B and Table 4). The regression coefficients (r) are greater than 0.993, indicating that the applicability of the method to the data is excellent. The median-lethal doses (LD_{50}'s) for rotenone, pyrethrins and their mixture (1:5) as calculated from the median-effect plot are 0.157, 0.916 and 0.450 mg/cc, respectively (Fig. 9B). These values are in close agreement with those obtained from probit analysis by Finney (9) who obtained 0.156, 0.918 and 0.455 mg/cc, respectively.

The original authors interpreted the results as indicating no striking antagonistic or synergistic effect of the mixture. Richardson used a predictive method for the mixture equivalent to the similar action law (9) and asserted that there was pronounced synergism. Bliss supported Richardson's conclusion, and Finney, after a new analysis of data, also agreed that there

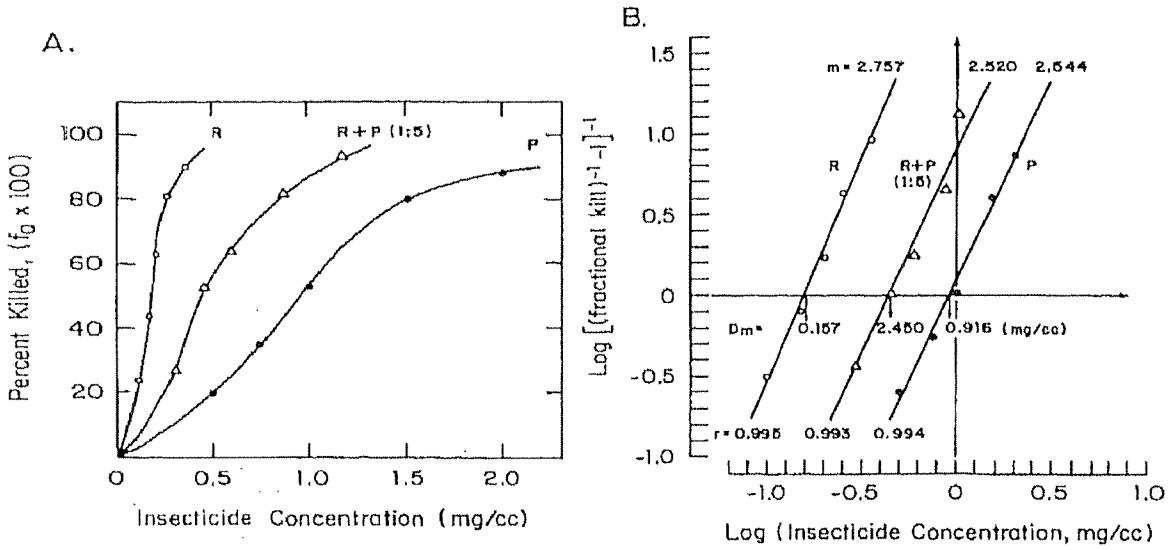

FIG. 9. Lethality of rotenone and pyrethrins to houseflies. Experimental data of LePelley and Sullivan (60), retrieved in ref. 13, were plotted for rotenone (R), pyrethrins (P), and their mixture (1:5, by weight) (R + P), on an arithmetic scale (*A*), and according to the median effect plot (*B*).

TABLE 4. TOXICITY OF ROTENONE AND PYRETHRINS TO HOUSEFLIES (60)

Insecticide	Parameters of median effect equation			
	m	D_m (mg/cc)	$y_{intercept}$	r
Rotenone	2.757 (± 0.157)	0.1571	2.216	0.9952
Pyrethrins	2.528 (± 0.158)	0.9097	0.0964	0.9960
Rotenone-Pyrethrins mixture (1:5)	2.519 (± 0.162)	0.4497	0.8743	0.9938

The m values (± S.E.) are the slopes of plots of $\log[(f_a)^{-1} - 1]^{-1}$ with respect to log (D), and were obtained by simple regression analysis on a programmable electronic calculator which also gives the y-intercepts and the linear regression coefficients (r). The median effect concentration is given by antilog (–y-intercept/m). These results may also be obtained by the use of a computer program developed for this purpose (37).

was evidence of synergism (9). The present paper uses a new method and shows that rotenone and pyrethrins are indeed somewhat synergistic, as shown quantitatively in Figure 10.

SUMMARY

A generalized method for analyzing the effects of multiple drugs and for determining summation, synergism and antagonism has been proposed. The

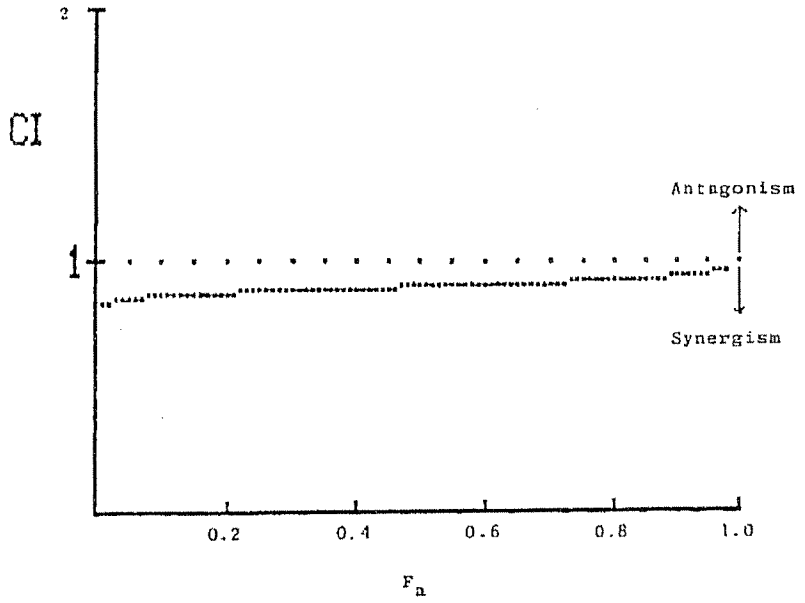

FIG. 10. Computer-generated graphical presentation of the combination index (CI) with respect to fraction affected (f_a) for the lethality to houseflies of a mixture of rotenone and pyrethrins (ratio of 1:5, by weight). The data of ref. 13 (Fig. 9) and the method of calculation described in the legend to Fig. 3 were used.

derived, generalized equations are based on kinetic principles. The method is relatively simple and is not limited by 1) whether the dose–effect relationships are hyperbolic or sigmoidal, 2) whether the effects of the drugs are mutually exclusive or nonexclusive, 3) whether the ligand interactions are competitive, noncompetitive or uncompetitive, 4) whether the drugs are agonists or antagonists, or 5) the number of drugs involved.

The equations for the two most widely used methods for analyzing synergism, antagonism and summation of effects of multiple drugs, the isobologram and fractional product concepts, have been derived and been shown to have limitations in their applications. These two methods cannot be used indiscriminately. The equations underlying these two methods can be derived from a more generalized equation previously developed by us (59). It can be shown that the isobologram is valid only for drugs whose effects are mutually exclusive, whereas the fractional product method is valid only for mutually nonexclusive drugs which have hyperbolic dose–effect curves. Furthermore, in the isobol method, it is laborious to find proper combinations of drugs that would produce an iso-effective curve, and the fractional product method tends to give indication of synergism, since it underestimates the summation of the effect of mutually nonexclusive drugs that have sigmoidal dose–effect curves. The method described herein is devoid of these deficiencies and limitations.

The simplified experimental design proposed for multiple drug-effect analysis has the following advantages: 1) It provides a simple diagnostic plot (i.e., the median-effect plot) for evaluating the applicability of the data, and provides parameters that can be directly used to obtain a general equation for the dose–effect relation; 2) the analysis which involves logarithmic conversion and linear regression can be readily carried out with a simple programmable electronic calculator and does not require special graph paper or tables; and 3) the simplicity of the equation allows flexibility of application and the use of a minimum number of data points. This method has been used to analyze experimental data obtained from enzymatic, cellular and animal systems.

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APPENDIX

DERIVATION OF FRACTIONAL PRODUCT EQUATION AND ISOBOLOGRAM EQUATION. COMPARISON OF APPLICABILITY AND LIMITATIONS OF THESE METHODS

Derivation of Fractional Product Equation

Many investigators have assumed that the summation of the effects of two inhibitors can be expressed by the product of the fractional activities, i.e. $(f_u)_{1,2} = (f_u)_1 \times (f_u)_2$, as formalized by Webb (42) on intuitive grounds or assuming independence of inhibitor action.

Since $f_a = 1 - f_u$, equation 3 can be simplified (surprisingly) to:

$$(f_u)_{1,2} = (f_u)_1 \times (f_u)_2$$

or

$$[1 - (f_a)_{1,2}] = [1 - (f_a)_1]\,[1 - (f_a)_2] \tag{11}$$

It is, therefore, clear that the fractional product equation describes only mutually nonexclusive first-order behavior.

Limitations of Fractional Product Method

The widely used fractional product concept of Webb (see equation 11) has serious limitations when applied to the analysis of the effect of multiple drugs. This method does not take into consideration the possible sigmoidicity of the dose–effect curve and the case where the drugs are mutually exclusive. The sample calculations shown in Table 5 illustrate the fact that the fractional product method is valid with respect to the present method only when the following conditions are satisfied: 1) D_1 and D_2 are mutually nonexclusive drugs, and 2) the dose–effect relationships of both D_1 and D_2 follow Michaelis–Menten-type hyperbola (i.e., $m = 1$).

As shown in Table 5, when the dose–effect curve is sigmoidal (e.g., $m > 1$), the fractional product method grossly underestimates the combined effect for mutually nonexclusive drugs and, in some cases, mutually exclusive drugs and, thus, may lead to false claims of synergism. In the case where the dose–effect curve is inversely sigmoidal (i.e., $m < 1$), the fractional product method will overestimate the combined effect, and thus lead to false claims of antagonism. However, the inverse sigmoidicity which is equivalent to negative cooperativity in enzyme systems is a relatively rare phenomenon. The aforementioned underestimation or overestimation of combined effects is particularly prominent at low f_u values. Table 5 also indicates that mutually nonexclusive drugs produce greater combined effects than mutually exclusive drugs at any level of sigmoidicity of the dose–effect curves.

TABLE 5. COMPARISON OF PREDICTIONS OF COMBINED EFFECTS OF MUTUALLY NONEXCLUSIVE AND EXCLUSIVE DRUGS ACCORDING TO THE FRACTIONAL PRODUCT (WEBB) METHOD AND THE PRESENT METHOD

Sigmoidicity of dose-effect curve for D_1 and D_2 (m value)	Effect level compared (when $(f_a)_1$ and $(f_a)_2$ are equal)	Combined effect, $(f_a)_{1,2}$, predicted by		
		Fractional product method*	Present method for mutually nonexclusive drugs†	Present method for mutually exclusive drugs§
0.5‡	0.05	0.0975	0.06932	0.06928
	0.1	0.19	0.1362	0.1358
	0.3	0.51	0.3878	0.3774
	0.5	0.75	0.6340	0.5858
	0.7	0.91	0.8643	0.7674
	0.9	0.99	0.9880	0.9272
1	0.05	0.0975	0.0975	0.0952
	0.1	0.19	0.19	0.1818
	0.3	0.51	0.51	0.4615
	0.5	0.75	0.75	0.6667
	0.7	0.91	0.91	0.8235
	0.9	0.99	0.99	0.9474
2	0.05	0.0975	0.2074	0.1739
	0.1	0.19	0.3769	0.3077
	0.3	0.51	0.7513	0.6316
	0.5	0.75	0.9	0.8
	0.7	0.91	0.9667	0.9032
	0.9	0.99	0.9956	0.9730
3	0.05	0.0975	0.4134	0.2963
	0.1	0.19	0.6291	0.4706
	0.3	0.51	0.8995	0.7742
	0.5	0.75	0.9643	0.8889
	0.7	0.91	0.9885	0.9492
	0.9	0.99	0.9984	0.9863

*The fractional product concept depicted by equation 3 or 11. This method does not take into consideration the sigmoidicity of dose-effect curves and the exclusivity of effects of drugs.

†The median-effect principle of multiple drugs depicted by equation 5.

§The median-effect principle of multiple drugs depicted by equation 4.

‡Inverse sigmoidicity ($m < 1$) is equivalent to negative cooperativity and is a relatively rare phenomenon.

Derivation of ED_{50} Isobol Equations

The isobol or isobologram for representing the equi-effective graph of the combination of two drugs at their various doses was previously proposed by Loewe (40, 41) for analyzing synergism, summation and antagonism of effects.

It may be seen from equations 2–5 that, for the special case, when $(f_a)_{1,2} = 0.5$, the above relationships are all equal to 1 and hence the magnitude of the values of m (i.e. the sigmoidicity of the dose–effect curve) for the drugs is

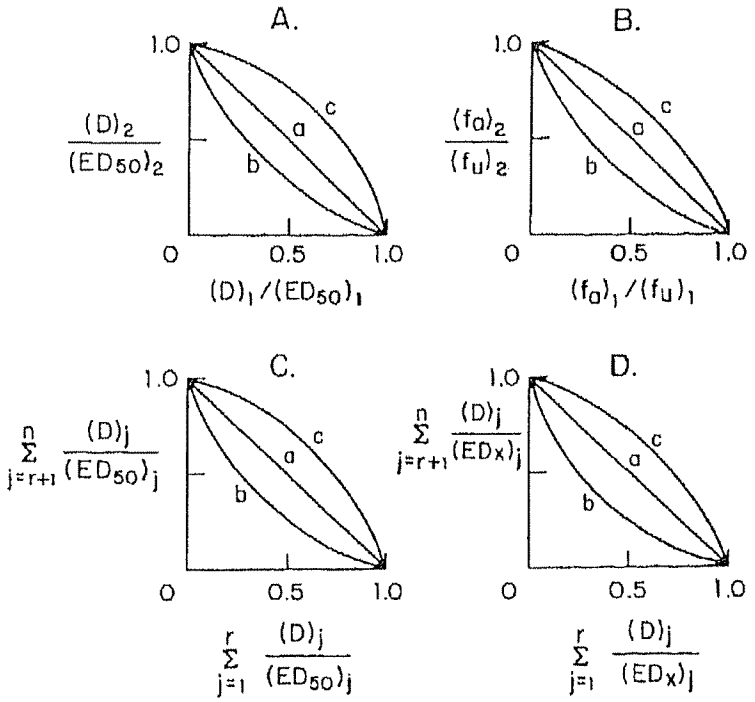

FIG. 11. Theoretical isobols for two or more drugs whose effects are mutually exclusive. *A* and *B*, ED_{50}-isobols for two drugs, *C*, ED_{50}-isobol for n drugs, and *D*, ED_x-isobol for n drugs. The straight lines, a, in isobolograms *A*, *B*, and *C* are based on equations 6, 12, and 20, respectively, and the straight line, a, in *D* is based on equation 21. In all cases, the additive iso-effective plot gives a straight line. If a concave upward curve or data point located below the straight line occurs (e.g. For *A*, curve b, $[(D)_1/(ED_{50})_1] + [(D)_2/(ED_{50})_2] < 1$), then synergism is indicated. If a convex downward curve or data point located above the straight line occurs (e.g., for *A*, curve c, $[(D)_1/(ED_{50})_1] + [(D)_2/(ED_{50})_2] > 1$), then antagonism is indicated. For mutually nonexclusive drugs, the isobologram cannot be used, since the equation contains a third term (see equations 3, 5 and 7). The limitations in using the isobologram are discussed in the Appendix.

irrelevant. For instance, equation 2 or 4 (for mutually exclusive drugs) become the equation which describes Loewe's isobologram (Fig. 11A):

$$\frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} = 1 \tag{6}$$

which can now be extended to (Fig. 11B):

$$\frac{(f_a)_1}{(f_u)_1} + \frac{(f_a)_2}{(f_u)_2} = 1 \tag{12}$$

where the two terms on the left can be assigned to the x and y axes, and equation 3 (for mutually nonexclusive drugs) becomes:

$$\frac{(f_a)_1}{(f_u)_1} + \frac{(f_a)_2}{(f_u)_2} + \frac{(f_a)_1\,(f_a)_2}{(f_u)_1\,(f_u)_2} = 1 \tag{13}$$

or

$$\frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} + \frac{(D)_1\,(D)_2}{(ED_{50})_1\,(ED_{50})_2} = 1 \tag{7}$$

Since there is an additional term in equation 7 or 13 when compared with equation 6 or 12, Loewe's isobologram is valid for mutually exclusive drugs but is not valid for mutually nonexclusive drugs.

Derivation of Generalized Isobol Equation

For the ED_x-isobol for any degree of effect, the general equation for summation for mutually exclusive agents is:

$$\frac{(D)_1}{(ED_x)_1} + \frac{(D)_2}{(ED_x)_2} = 1 \tag{8}$$

where $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 alone, respectively, that give x percent affected, and $(D)_1$ and $(D)_2$ (in the numerators) in combination give x percent affected [i.e., $(D_x)_{1,2} = (D)_1 + (D)_2$].

Equation 8 is valid irrespective of the m value and irrespective of the dose ratio of D_1 and D_2 that, in combination, would produce an f_a value of x percent. Equation 8 can be derived as follows:

If we assume: 1) D_1 and D_2 are mutually exclusive drugs
2) D_1 and D_2 both follow m^{th} order kinetics
3) Dose ratio of D_1 and D_2 in combination is 1:a (i.e., $D_2 = aD_1$)
4) D_1 and D_2 in combination affect a target system x percent which produces $[(f_a)_x]_{1,2}$.

From equation 4, for two drugs in combination, we obtain:

$$\left\{\frac{[(f_a)_x]_{1,2}}{1-[(f_a)_x]_{1,2}}\right\}^{\frac{1}{m}} = \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} = \frac{(D)_1}{(ED_{50})_1} + \frac{a(D)_1}{(ED_{50})_2}$$

$$= \frac{(D)_1\,(ED_{50})_2 + a(D)_1\,(ED_{50})_1}{(ED_{50})_1\,(ED_{50})_2} \tag{14}$$

Let the term on the left be represented by A (i.e., $A = \{[(f_a)_x^{-1} - 1]^{-1}\}^{1/m}$. Then:

$$(D)_1 = \frac{A(ED_{50})_1\ (ED_{50})_2}{a(ED_{50})_1 + (ED_{50})_2} \tag{15}$$

$$(D)_2 = \frac{aA(ED_{50})_1\ (ED_{50})_2}{a(ED_{50})_1 + (ED_{50})_2} \tag{16}$$

When each drug alone affects a target system x percent (i.e. $(ED_x)_1$ produces $[(f_a)_x]_1$ and $(ED_x)_2$ produces $[(f_a)_x]_2$, then, from equation 1, we obtain:

$$\left\{\frac{[(f_a)_x]_1}{1 - [(f_a)_x]_1}\right\}^{\frac{1}{m}} = \frac{(ED_x)_1}{(ED_{50})_1} \tag{17}$$

and

$$\left\{\frac{[(f_a)_x]_2}{1 - [(f_a)_x]_2}\right\}^{\frac{1}{m}} = \frac{(ED_x)_2}{(ED_{50})_2} \tag{18}$$

At an iso-effective condition, $[(f_a)_x]_{1,2}$, $[(f_a)_x]_1$ and $[(f_a)_x]_2$ are all equal (i.e., equal to A, or $\{[(f_a)_x^{-1} - 1]^{-1}\}^{1/m}$). Thus, $(ED_x)_1 = A(ED_{50})_1$ and $(ED_x)_2 = A(ED_{50})_2$, and thus:

$$\frac{(D)_1}{(ED_x)_1} + \frac{(D)_2}{(ED_x)_2} = \frac{\dfrac{A(ED_{50})_1\ (ED_{50})_2}{[a(ED_{50})_1 + (ED_{50})_2]}}{A(ED_{50})_1} + \frac{\dfrac{aA(ED_{50})_1\ (ED_{50})_2}{[a(ED_{50})_1 + (ED_{50})_2]}}{A(ED_{50})_2}$$

$$= \frac{(ED_{50})_2}{a(ED_{50})_1 + (ED_{50})_2} + \frac{a\,(ED_{50})_1}{a(ED_{50})_1 + (ED_{50})_2}$$

$$= 1$$

Therefore, equation 8 is confirmed.

Therefore, irrespective of the magnitude of the m value and irespective of the ratio of doses in the combination (a), equation 8 for the ED_x-isobologram is always valid for mutually exclusive drugs. $(ED_x)_1$ and $(ED_x)_2$ in the numerator of equations 17 and 18, or the denominator of equation 8 can be readily obtained from equation 1 or the median-effect plot. $(ED_x)_{1,2}$ that produces the iso-effect can also be obtained from equation 1. $(ED_x)_{1,2}$ can then be dissected into its components $(D)_1$ and $(D)_2$ [i.e., $(ED_x)_{1,2} = (D)_1 + (D)_2$]. Since the ratio of $(D)_1$ and $(D)_2$ in the mixture is known at the outset of the experiments [e.g. $(D)_1/(D)_2 = P/Q$], $(D)_1$ and $(D)_2$ in the numerators of equation 8 can be obtained by $(D)_1 = (ED_x)_{1,2} \times P/(P+Q)$ and $(D)_2 = (ED_x)_{1,2} \times Q/(P+Q)$.

Therefore, the CI procedures described above, as depicted in equations 9 and 10, eliminate the labor and the limitations of the old isobologram method.

The present study also allows additional new isobolograms to be formulated:

For mutually exclusive drugs, the isobol for the combination of n inhibitors (59) can be described by:

$$\sum_{j=1}^{n} \frac{(f_u)_j}{(f_u)_j} = 1 \tag{19}$$

for the ED_{50}-isobologram, or by (Fig. 11C)

$$\sum_{j=1}^{n} \frac{(D)_j}{(ED_{50})_j} = 1 \tag{20}$$

which can now be extended to:

$$\sum_{j=1}^{n} \frac{(D)_j}{(ED_x)_j} = 1 \tag{21}$$

for a generalized isobologram (see Fig. 11D).

The n can be partitioned into any combination of two parts (e.g., $\sum_{j=1}^{n} = \sum_{j=1}^{m} + \sum_{j=m+1}^{n}$ where $1 \leqslant m \leqslant n-1$, m and n are integers) which can be assigned to the two coordinates of the isobol. Thus, a three-dimensional isobologram of great complexity (41, 45) will not be necessary, and the three-dimensional presentation of effects (45, 48) can be extended to four or more drugs and yet use only two-dimensional graphs.

Limitations of the Isobologram Method

Loewe's isobologram (40, 41) is valid for equations 8 or 12 but not for equation 7 or 13. Thus, mutually nonexclusive inhibitors (which are in fact additive in equation 13) would appear to be slightly synergistic in Loewe's isobologram.

Aside from the limitation of the isobologram and its applicability only to mutually exclusive drugs, the major practical drawback of the isobol method is that it is laborious to find proper combinations of drugs that would produce an iso-effect point. In all cases, general equations (equations 4 and 5 or 9 and 10) can be more conveniently used than the isobolograms since the latter are merely special cases of equation 4.

EXHIBIT D

Perspective

Cancer Research

Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method

Ting-Chao Chou

Abstract

This brief perspective article focuses on the most common errors and pitfalls, as well as the do's and don'ts in drug combination studies, in terms of experimental design, data acquisition, data interpretation, and computerized simulation. The Chou-Talalay method for drug combination is based on the median-effect equation, derived from the mass-action law principle, which is the unified theory that provides the common link between single entity and multiple entities, and first order and higher order dynamics. This general equation encompasses the Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations in biochemistry and biophysics. The resulting combination index (CI) theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations. This theory also provides algorithms for automated computer simulation for synergism and/or antagonism at any effect and dose level, as shown in the CI plot and isobologram, respectively. *Cancer Res; 70(2): 440–6. ©2010 AACR.*

Introduction

Drug combination is most widely used in treating the most dreadful diseases, such as cancer and AIDS. The main aims are to achieve synergistic therapeutic effect, dose and toxicity reduction, and to minimize or delay the induction of drug resistance (1). Toxicity reduction and resistance minimization benefits could also be the outcomes of synergism. However, in one review article by Goldin and Mantel in 1957 (2), seven different definitions for synergism were given, and in a more recent review by Greco and colleagues in 1995 (3), 13 different methods for determining synergism were listed and none of them supported the others (1). The meaning of synergism has become an individual's preference. Faulty or unsubstantiated synergy claims are pervasive. This is serious because it is frequently referred to in patient therapy.

Without a standardized definition for synergism, it is argued that there will be a mess in making synergy claims, whether in publishing a scientific article, submitting a grant application, planning drug combination clinical trials for Food and Drug Administration approval, or asserting drug combination discovery to the Patent Office (4, 5). It is also argued that in the absence of a clear "definition for synergism", governmental agencies have no basis to regulate the drug combination synergy claims (4, 5).

I have devoted over four decades on this important fundamental issue. In all, more than 300 mechanism-specific equations have been derived and published (6–13). It took about 10 years to figure out what an additive effect is (1, 7, 12). This is important, because by definition, synergism is more than an additive effect and antagonism is less than an additive effect.

Along with Professor Paul Talalay of the Johns Hopkins University School of Medicine, in 1983 to 1984, we jointly introduced a scientific term "combination index" (CI) to quantitatively depict synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1; refs. 11, 12). Its applications were greatly facilitated by the help of Joseph Chou, who developed the first-generation computer software for dose-effect analysis based on the "mass-action law" (14). Due to fast changes in computer hardware and software, the second-generation "CalcuSyn" was written by Mike Hayball of Cambridge, United Kingdom in 1997 (15), and the third-generation "CompuSyn" was then written by Nick Martin of MIT, Cambridge, MA in 2005 (16).

The route from the unified theory to algorithms, to quantitative practical applications is shown in Fig. 1. The essence behind the mass-action law–based theory is that the "median" is the unified common link of single entity and multiple entities, and for the first-order and higher-order dynamics (1). The "median" in the median-effect equation also serves as the universal reference point, which evolves into the global positioning system (GPS) concept for bioinformatics. The features of this methodology are its simplicity and flexibility (e.g., mechanism- and unit-independent), its quantitative definition (e.g., numerically indexed conclusion), and its efficiency and economy (e.g., allows for a small number of data points of measurements and uses a small number of animals). More details have been described in refs. 1 and 17.

The Common Pitfalls and Errors in Drug Combination Studies

To avoid pitfalls in drug combination studies, the following fundamental, scientific concept, and practical issues need

Author's Affiliation: Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, New York

Corresponding Author: Ting-Chao Chou, Preclinical Pharmacology Core Laboratory, Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: 212-639-7480; Fax: 212-794-4342; E-mail: chout@mskcc.org.

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to be addressed. Below are my scientific views (1, 4, 5). Debates are welcome because there is an urgent need for synergy definition that requires a global consensus and standardization.

1. ***The nature of the problem.*** Synergism is basically a physicochemical mass-action law issue, not a statistical issue. Determine synergism with CI values, not with *P* values.
2. ***The P value?*** A combined effect greater than each drug alone does not necessarily indicate synergism. Sometimes this can be a result of additive effect or even a slight antagonism. A + B > A or A + B > B is a simple axiom which does not require elaborate proof, such as *P* values. Thus, if the combined effect is greater than each drug alone, it does not necessarily indicate synergism.
3. ***The arithmetic sum.*** The additive effect of two drugs is not the simple "arithmetic sum" of effects of two drugs. If $(D)_1$ and $(D)_2$ inhibits 30% and 40%, respectively, the additive effect is not 70%, because if they inhibit 60% and 70%, respectively, the additive effect cannot be 130%!
4. ***The fractional product concept.*** If $(D)_1$ and $(D)_2$ each inhibit 50%, then the combined additive effect is 75%. Is this correct? Answer: it is most likely wrong, because it is valid only when the dose-effect curves for both drugs are hyperbolic [i.e., follows the Michaelis-Menten (first order) kinetics/dynamics with $m = 1$; ref. (18)] and only if the effects of both drugs are mutually nonexclusive (1, 10). In simple enzyme or receptor systems, where m is frequently ≈ 1, we can calculate additive effect by $(1 - 0.5) \times (1 - 0.5) = 0.25$, and $(1 - 0.25) = 0.75$ as indicated by Webb (19). However, Chou and Talalay indicate that in cellular or animal systems, the dose-effect curves are most likely sigmoidal ($m > 1$) or flat sigmoidal ($m < 1$; refs. 1, 12, 13). When we talk about the dose-effect curve, we need to take into account both the potency (the D_m or IC_{50} value) and the shape of the dose-effect curve (the m value) simultaneously, not just pay attention to potency.
5. **In vitro *versus in animals.*** Does the determination of synergy *in vitro* and in animals follow the same principle? The answer is yes. The main practical differences in animal drug combinations are (*a*) it is more expensive, (*b*) it is more time-consuming, (*c*) there is more variability, and (*d*) the smaller population size (i.e., smaller n). For anticancer drug combination studies against xenograft tumors in nude mice under optimal therapeutic conditions, only 65 nude mice were used in Chou's laboratory to determine synergy with F_a-CI plot and isobologram (5, 20, 21). By contrast, using the empirical response surface method (3), one needs to use ~800 animals, and yet, the synergy conclusion may still be vague and not quantitative. The determined synergism or antagonism is obtained from the given experimental conditions. The extrapolation of results from *in vitro* to animals, or from animals to humans, is a general and separate biomedical problem which is not expected to be solved by the Chou-Talalay method.
6. ***Determining synergy in clinics?*** Is it possible to "determine" synergism in clinical trials or in clinics? The answer is generally no for the disease per se (e.g., cancer or AIDS). This is based on scientific, practical, and ethical reasons, as indicated in ref. 1 (pp. 641–642) and in ref. 5 (Table 1). Most clinical synergy claims thus far, to my knowledge, are not supported by the available data, especially when only a single dose for a single drug was used. This is why, prior to drug combination clinical trials, preclinical drug combination studies *in vitro* and/or in animals should be carried out to obtain the basis and rationale for studies in humans. To obtain the therapeutic benefits of drug combination in humans, the basis and rationale involving explorative details should be obtained from preclinical studies and should not be done entirely on human subjects. This is the principle that the Food and Drug Administration needs to enforce. When two drugs are combined, they can behave like a third drug with a lot of uncertainties. It should be noted that by using a surrogate marker and the repeated fractional-dose schedule of multiple doses, it is possible to determine synergy in a small-scale clinical trial. An elegant example is using P24 antigen or CD_4^+ as a marker, synergy with CI value can be obtained in AIDS clinical trials for AZT and IFNα using only 36 patients (22). In another AIDS clinical trial for AZT and 3TC (23), 366 patients were used, also using surrogate markers such as CD_4^+ and HIV-RNA; however, this study could not determine synergy because only a single dose of AZT was used. It only concluded that combined therapy was better than monotherapy alone, which is equivalent to A + B > A or A + B > B, which is not the definition for synergy.
7. ***Prediction by mechanisms?*** Is a knowledge of mechanisms required for determining synergism? Answer: no, because the mass-action law–based determination of synergism is mechanism-independent. Information about the mechanism of action is good to have for knowledge and for guesswork. In reality, there are many well-known drugs whose various mechanisms we know very little about, for example, aspirin which has been widely used for over a century. In most cases, we cannot predict synergy from mechanisms, e.g., taxol (microtubule stabilization) + cisplatin (DNA alkylation) + topotecan (DNA topoisomerase I inhibition; ref. 1; Table 10 and ref. 24, Fig. 1). Even if it is partially predictable, it is still not quantitative, e.g., taxol + MDR-reversing agent, such as ningalin (25) or anti-HIV agents, ribavirin + zidovudine (26). In addition, some drugs have several modes of actions (e.g., upregulate or downregulate a lot of gene expressions) and it is difficult to determine which mode of action contributed to the synergy and to what extent. Furthermore, there is an issue of efficiency of research that can be raised because the quantitative determination of synergism of two drugs *in vitro* usually takes 1 to 2 weeks, but to figure out how and why synergy occurs may take several years and the conclusion could

still likely be a "might be", "maybe", "suggest", "imply", etc. (1, 5). Synergy or antagonism needs to be determined, not to be predicted. In this case, to determine is easy but to predict is difficult. If synergy is predictable, then there would be no need to conduct drug combination studies. Sometimes, the prediction might be correct by luck but it will not be quantitative. Frequently, predictions were done after the observed facts retrospectively, as can be seen in the biomedical literature. There has been no rigorous theory or method to predict synergy, except that "polygonogram", introduced by Chou and Chou (1, 27), can frequently project synergism or antagonism semiquantitatively (see Fig. 9 in ref. 1). This powerful and efficient projection, however, still needs a certain amount of experimental work (e.g., project the outcomes of three-drug or four-drug combinations based on two-drug combinations for the semiquantitative projection of outcomes).

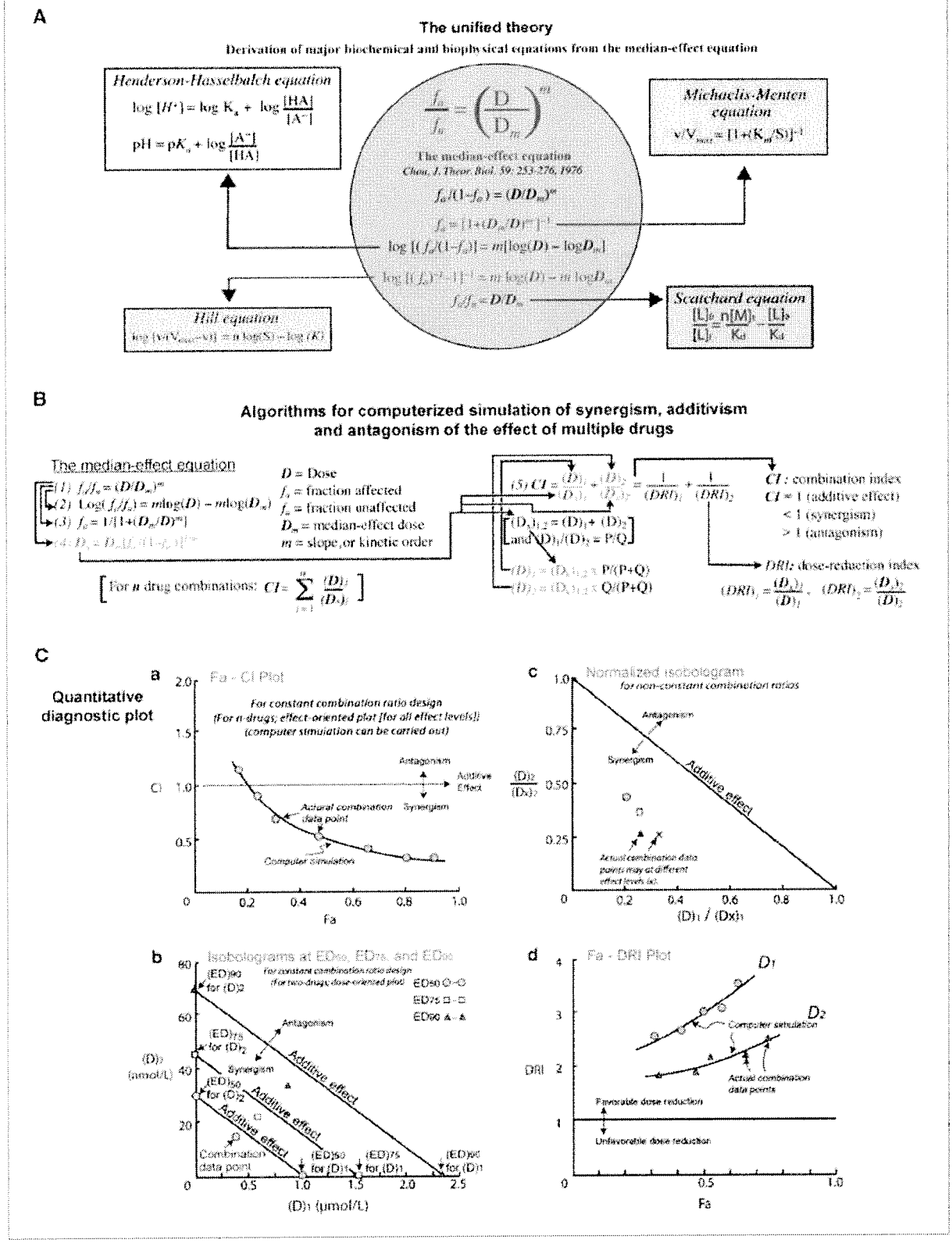
8. ***Synergism versus enhancement.*** Does synergism and enhancement have the same meaning? Answer: no. Synergism (or antagonism) is "mutual" whereas enhancement, potentiation, or augmentation is "one-sided" (1, 5). Synergism or antagonism needs to be determined with CI values, whereas for enhancement, potentiation, or augmentation, we simply just need to state x% potentiation or y-fold enhancement, etc. When a drug but itself has no effect, there will be no D_m and m values for calculating CI.
9. ***Efficiency and economy.*** Based on Chou's theory, is it possible to draw a specific curve with only two data points? Answer: yes, if they are accurately determined (1, 5, 17). This statement defies the widely held belief that from two data points, we can only draw a straight line. Here, the two points is actually four points. The third point is the origin at dose zero, and the fourth point is the median, which is the common link and universal reference point for the first-order and higher order kinetics/dynamics, as well as for the single entity and multiple entities (1, 5, 17). When the mass-action law parameters (D_m for potency and m for shape) are determined by the median-effect plot (Chou plot), the entire dose-effect curve is automatically determined (e.g., using CompuSyn software simulation; ref. 16). This is the basis for the GPS concept for bioinformatics proposed by Chou. This concept should have far-reaching consequences in biomedical research, in terms of efficiency and economy, and in the conservation of human workforce, materials, and animal resources. This is exemplified in the CI method for drug combination studies, discussed in this brief perspective article. More details are given in refs. (1, 5, 17).

Frequently Asked Questions for the CI Method and "Do's and Don'ts"

Given below are frequently asked questions for the Chou-Talalay CI method, in terms of experimental design, data entry, data analysis with CompuSyn software, and other practical questions. I will try to give answers clearly, but briefly. For more details, please refer to refs. 1 and 5. For simplicity, the following discussions refer to two-drug combinations *in vitro*. The questions or issues are itemized below for easy references, in a random order.

1. ***Why do you recommend the constant-ratio drug combinations?*** The cells do not know it is a single drug or drug combination, nor its combination ratio, nor their mechanisms, etc. We just enter the "dose" and "effect" for each drug and combination's numerical information to the computer. When we make a mixture and dilute it serially (usually 2-fold serial dilution with several concentration points above and below its IC_{50} value), it will always stay at a constant ratio [at the $(IC_{50})_1/(IC_{50})_2$ ratio or other selected ratio; refs. (1, 5), if you have reason(s) to do so, such as solubility limit or intend to de-emphasize one drug over others to avoid bad toxicity or side effects; refs. (1, 5, 12, 28)]. This mixture, in fact, behaves like a third drug to the cells. In this way, we can obtain the parameters [$(D_m)_{1,2}$, $(m)_{1,2}$ and $(r)_{1,2}$ for the mixture, just like the single drugs $(D_m)_1$, $(m)_1$, $(r)_1$ and $(D_m)_2$, $(m)_2$, $(r)_2$] by using the automated median-effect plot (the Chou plot) with a computer software. The r value is the linear correlation coefficient of the median-effect plot, which signifies the conformity of the data to the mass-action law. For *in vitro* experiments, it usually yields $r > 0.97$. The advantage is that they allow the automated computer simulation for the F_a-CI plot (CI plot or Chou-Talalay plot), F_a-DRI plot (dose-reduction index plot or Chou-Martin plot), and the classic isobologram (and if there are more than two drugs, the polygonogram or Chou-Chou graphics), in addition to the dose-effect curves and the median-effect plot. If the experiment is carried out in non-constant ratio combinations (e.g., 1:1, 1:2, 1:5, and 1:10), the CI values can still be determined at their corresponding specified data points,

Figure 1. The route from theory to algorithms, to practical applications for drug combination studies. *A*, the median-effect equation as the unified theory. *B*, the merging of the median-effect equation with the CI equation leads to the quantitative definition for synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1), and provides the algorithms for their computer simulation. Rearrangement of the equation also provides algorithms for simulation of dose-reduction index (*DRI*) for each drug in their combination. The favorable DRI (>1) allows dose-reduction that leads to toxicity reduction in the therapeutic applications. *C*, the depiction of typical quantitative diagnostic graphics generated by the computer simulation. *a*, the F_a-CI plot (Chou-Talalay plot). *b*, the classic isobologram (for the constant ratio combination design). *c*, the normalized isobologram (Chou-Chou plot) for the non-constant ratio combination design. *d*, the F_a-DRI plot (Chou-Martin plot) for the constant ratio combination design. When all single drug parameters (m and D_m values) are available, the CI values can be calculated for the non-constant ratio combinations. But no computer simulation for F_a-CI plot or the F_a-DRI plot is possible due to changing ratios (*A* is reproduced from ref. 13 with permission from Elsevier. *B* and *C* are reproduced from ref. 1 with permission from the American Society of Pharmacology and Experimental Therapeutics).


A
The unified theory
Derivation of major biochemical and biophysical equations from the median-effect equation
Henderson-Hasselbalch equation
log [H+] = log Ka + log [HA]/[A−]
pH = pKa + log [A−]/[HA]
The median-effect equation
Chou, J. Theor. Biol. 39: 253-276, 1976
Michaelis-Menten equation
Hill equation
Scatchard equation
B
Algorithms for computerized simulation of synergism, additivism and antagonism of the effect of multiple drugs
The median-effect equation
D = Dose
fa = fraction affected
fu = fraction unaffected
Dm = median-effect dose
m = slope, or kinetic order
For n drug combinations:
CI: combination index
CI = 1 (additive effect)
< 1 (synergism)
> 1 (antagonism)
DRI: dose-reduction index
C
Quantitative diagnostic plot
a
Fa - CI Plot
For constant combination ratio design
(For n drugs; effect-oriented plot [for all effect levels])
(computer simulation can be carried out)
Antagonism
Additive Effect
Synergism
Actural combination data point
Computer simulation
CI
Fa
b
Isobolograms at ED50, ED75, and ED90
For constant combination ratio design
(For two-drugs; dose-oriented plot)
ED50 ○-○
ED75 □-□
ED90 △-△
Antagonism
Synergism
Additive effect
Combination data point
(D)1 (μmol/L)
(D)2 (μmol/L)
c
Normalized isobologram
for non-constant combination ratios
Antagonism
Synergism
Additive effect
Actual combination data points may at different effect levels (e)
(D)2 / (D)1
(Dx)2 / (Dx)1
d
Fa - DRI Plot
D1
D2
Computer simulation
Actual combination data points
Favorable dose reduction
Unfavorable dose reduction
DRI
Fa

if the $(m)_1$, $(D_m)_1$ and $(m)_2$, $(D_m)_2$ for single drugs are available, but no "simulation" for the CI plot. In nonconstant ratio design, the DRI plot can be done for specific data points but with no simulations, and the computer-generated classic isobologram (usually from the constant-ratio combinations) will be replaced with the computer-generated "normalized isobologram" (or the Chou-Chou conservative isobologram; refs. 1, 26). Typical representations of the classic and conservative isobolograms are shown in Fig. 1*C* (and in Fig. 9 of ref. 1 or Fig. 5 of ref. 5). If one prefers to have more information of more than one combination ratio, and if the experimental size and cost are of less concern (especially when facing animal studies), it is recommended to use a checkerboard or Latin square design, in which several constant-combination ratios are provided, as shown in Tables 5, 8, and 9 of ref. 1).

2. ***What is the prerequisite for all drug combination studies?*** Answer: the prerequisite is the dose-effect curves for each drug alone. Each drug not only has a different potency (the D_m value) but also a different shape of the dose-effect curve (the m value). For any determination of synergy, we need to know both the potency and the shape of the dose-effect curve of each drug (1, 5, 12). The D_m and m values can easily (and automatically) be obtained from the median-effect equation using computer software (e.g., CompuSyn) or by using a pocket calculator. For examples of manual calculations, see Table 10 in ref. 1. With these parameters [$(m)_1$, $(D_m)_1$ and $(m)_2$, $(D_m)_2$] available, we could determine whether there is synergism or antagonism, quantitatively, by using the CI equation, even for only a single combination data point (1). However, if multiple data points for constant-ratio combination are available [i.e., $(D_m)_{1,2}$ and $(m)_{1,2}$], then the entire spectrum of synergism or antagonism at all effect levels can be automatically simulated. Thus, the hypothetical minimum for a drug combination study is five data points, in which two are for $(D)_1$, two are for $(D)_2$, and one for the combination. For reasons of biological and technical variability, of course, we do not advocate the use of minimum data points. However, the median-effect principle (MEP) provides the legitimacy of using a small number of data points if variability is in a small magnitude (1, 5, 17).
3. ***Dose range and dose density questions.*** It is ideal to have several data points above IC_{50} and several below IC_{50} because this would make the assay more accurate. Because the unified theory of the median-effect equation and plot is general and versatile, it can easily handle screwed data points in which it's all above IC_{50} or all below IC_{50}, if the assays are accurate, and with a good r value (e.g., $r > 0.95$; refs. 1, 14–16). Although the dose range is very flexible for analytic purposes, it is important to realize whether the concentration range used *in vitro* is achievable *in vivo*, and whether these concentrations are within the tolerable toxicity *in vivo*. As a common rule, do not delete the data points in the middle of the dose-effect curve, unless there are specific reasons (e.g., inadvertent error or accident). However, at extremely high concentrations or at extremely low concentrations, where accurate assays are not possible (i.e., beyond the sensitivity of detection), the unreliable data points should be deleted (1). Otherwise, the computer software (based on the median-effect equation and plot) takes into account every data point equally significantly. Never enter 0% or 100% inhibition ($f_a = 0$ or $f_a = 1$) into the computer because log 0 = negative infinity (at infinitely low concentration) and log 1 = 0 (at infinitely high concentration) will lead the computer to crash (14–16).
4. ***What does extraordinarily high CI value mean?*** Sometimes the CI values are >3 or much greater, especially at low effect levels (i.e., low f_a level). Don't be surprised! Keep in mind that the synergy scale is from 1 to 0 and the antagonism scale is from 1 to infinity. Frequently, at high dose or high effect levels, the synergistic interaction is stronger than otherwise. For anticancer or antiviral agents, synergy at high effect levels (e.g., at $f_a > 0.8$) is more relevant to therapy than at low effect levels (e.g., at $f_a < 0.2$). A semiquantitative expression of CI ranges for synergism (or antagonism) in symbols, colors, and grades (slight, moderate, substantial, strong, and very strong) are recommended in Table 4 of ref. 1.
5. ***What is the scope of applications of the MEP and the CI method?*** The MEP of Chou (i.e., the median-effect equation and the median-effect plot; refs. 7 and 8) and the extended CI theorem of Chou-Talalay (i.e., the CI equation and plot; ref. 12) are derived from Nature's fundamental mass-action law in biophysics and biochemistry. The theory of MEP (for single entity) and of CI (for multiple entities) should be generally applicable in all dose-effect relationships that follow the mass-action law (i.e., with good r values; refs. 1, 17). The derivation of these equations is accomplished by using the mathematical induction/deduction from more than 300 mechanism-specific equations. As shown in refs. (7–10), the general equations are valid with different reaction mechanisms, different types of mechanisms of inhibition, and with different numbers of reactants.The broad application is attested by the fact that MEP and CI have been cited in more than 3,970 scientific articles globally, based on the Thompson ISI Web of Science search. It is of interest to note that one review article on drug combination by Chou and Talalay (12), which was published in a journal with an impact factor of 1.83, has now been cited in more than 1,915 scientific articles internationally from over 407 different biomedical journals. The CI method helps answer the following primary questions (1, 5): (*a*) Are there any synergisms? (*b*) How much synergism? (*c*) Synergism at what dose levels? (*d*) Synergism at what effect levels? (*e*) What did the exhibited CI plot, isobologram, and polygonogram look like? (*f*) How many folds of dose-reduction (for toxicity reduction) for each drug as a result of synergism? All of the above tasks could be accomplished with computerized simulation

for experiments, which normally takes ~1 to 2 weeks for the *in vitro* experiment to complete. For a slight alteration in experimental design, one can also answer these other questions: (*g*) the optimal combination ratio for maximal synergy, (*h*) the schedule dependency of synergy, (*i*) the selectivity of synergy against the target versus the host, and (*j*) the condition-directed synergism, such as the influence of pH, temperature, radiation, and oxygen tension, etc., on the synergistic outcome. The major advantages of this theory are its sound theoretical basis, general validity for broad application, and the algorithm for computer simulation. The overwhelming significance of the theory is its features of simplicity, efficiency, and economy, which have influenced the face of biomedical research in a measurable way. The validity of the MEP is, without a doubt, because it is the unified form of Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations, which are the main theories of biochemistry and biophysics (Fig. 1*A*). As indicated in refs. 1 and 17, the CI concept and its application is just an extension of the MEP. There are many other utilities on the horizon that can be applied to the median-effect equation and its principles (1), which include (*a*) conducting small-sized experimentations; (*b*) conservation of use of laboratory animals; (*c*) low-dose risk assessment of carcinogens, radiation, or toxic substances; (*d*) calculation of K_i from IC_{50}; (*e*) agricultural control of pests; (*f*) topologic analysis of exclusivity and competitiveness; (*g*) calculation of therapeutic index and safety margin; and (*h*) epidemiologic applications, such as age-specific disease incidences and their projections. These are just to mention a few. The overall significance of the median-mediated unity theory is its economic enabling of increasing efficiency for biomedical research, and the conservation of human workforce, materials, and animal resources. It also benefits the humanistic side on improving health and well-being by defining and determining synergism to avoid faulty synergism claims and confusions (1), and quickening the new drug discovery process by using the efficient quantitative approach for drug evaluation and the GPS concept for bioinformatics (17).

Conclusions and Future Directions

The CI is the natural law–based general expression of pharmacologic drug interactions. It is shown to be the simplest possible way for quantifying synergism or antagonism. Its simplicity in equations, experimental designs, and data analysis features efficiency, economy, and reducing the experimental size of animals used or the number of patients needed for drug combination clinical trials. The general theory of the MEP of the mass-action law, its CI algorithm, and its computerized simulation have paved the way for future drug combination studies, as indicated by the broad acceptance in scientific applications and by the rapid increase in citation numbers.

Disclosure of Potential Conflicts of Interest

T-C. Chou is a principal in and holds the copyright to CompuSyn, the software used for the analysis performed in this article. His son, who contributes to the software's development, is in a position to receive royalties for its publication. T-C. Chou has also received honoraria from seminars and lectures, and consultation fees from universities and pharmaceutical and biotech companies. The author has not received any grant supports for his four decades of theoretical work. The software development was financed by personal funds.

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